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Fort Detrick, Maryland 21702-5012

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14. ABSTRACT <p>Current endocrine therapy for ER+ve breast cancer involves Tamoxifen, and Aromatase inhibitors. De novo and/or acquired resistance to endocrine therapies however frequently occur. Interestingly, earlier studies have shown that activation of Cyclin Dependent Kinase 2 (CDK2) and deregulation of PELP1 (Proline, glutamic Acid and Leucine rich Protein), a novel ER coregulator is found associated with endocrine therapy resistance. The results of this study showed that PELP1 is a novel substrate of CDK2. Functional studies established PELP1 as a modulator of E2F transactivation function and PELP1 phosphorylation is needed for optimal activation of E2F target genes. Studies utilizing a novel PELP1 phospho antibody, model cell lines stably expressing the PELP1-shRNA or PELP1-CDK2 phospho-site mutants demonstrated that CDK phosphorylation of PELP1 is important for estrogen (E2) mediated cell cycle progression. Further, PELP1 phosphorylation by CDK is important for E2-mediated tumorigenesis in vivo and deregulation of CDK-PELP1 axis was observed in various endocrine resistant model breast cancer cells. Blocking CDK2 activation using Roscovitine, a drug currently in clinical trials, confers tumor suppressive effects. Further, Roscovitine has the potential to alter the ER alpha/beta ratio in breast cancer cells. Overall, these results suggest that CDK phosphorylation of PELP1 is important for E2 driven cell cycle progression and blocking CDK2-PELP1 axis activation by Roscovitine will have therapeutic implications.</p>					
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I. INTRODUCTION

The estrogen receptor (ER) plays a central role in the progression of breast cancer. Current endocrine therapy for ER+ve breast cancer involves modulating ER-pathway using Tamoxifen, (1) and blocking peripheral estrogen (E2) synthesis by Aromatase inhibitors (2). Despite the positive effects, *de novo* and/or acquired resistance to endocrine therapies frequently occur. Although mechanisms for hormonal therapy resistance remains elusive, emerging data implicate growth factor signaling pathways and its cross talk with ER as major cause of resistance (3-5). *Interestingly, most downstream events in these resistance signaling pathways converge upon modulation of cell cycle regulatory proteins; the most conspicuous of which is the upregulation of Cyclin E and A, along with activation of Cyclin Dependent Kinase 2 (CDK2)* ((6-10). Activation of CDK2 in these resistant tumors is reported to occur in part by downregulation of p27-CDK inhibitors (11-13) or in part by functional ablation of Retinoblastoma protein (pRb) and further upregulation of Cyclin E (14, 15). ER signaling complexes are known to recruit various co-regulatory proteins and recent evidences suggest that deregulated expression, localization and activity of ER coregulators also plays vital role in endocrine resistance (16); examples include SRC-1 (17), SRC3 (AIB1) (18-20), NCOR1 (21) and PELP1 (22). ***The major focus of this proposal is on PELP1*** (Proline, glutamic Acid and Leucine rich Protein), a novel ER coregulator (23, 24) normally expressed in mammary gland but shows deregulated expression and localization in breast tumors (25). Emerging studies implicate PELP1 as a proto-oncogene (26) and its deregulation might play a role in hormone therapy resistance (27). My preliminary results suggested that PELP1 is a novel substrate of CDK2. Based on these preliminary observations, I hypothesized that ***a) phosphorylation of PELP1 by CDK2 confers growth advantage to breast epithelial cells via upregulating ER/ E2F target genes and histone biosynthesis, thus contributing to its oncogenic potential; b) deregulation of CDK2-PELP1 signaling axis constitutes a novel signaling pathway towards acquired hormonal therapy resistance.***

II. BODY

The scope of this proposal is to undertake the following three tasks as outlined in the approved statement of work:

Task1. To determine the biological significance of CDK2 mediated phosphorylation of PELP1

Task2. To understand biological significance of CDK2 mediated phosphorylation of PELP1 on histone biosynthesis and PELP1's interactome

Task3. To investigate the therapeutic potential of CDK-PELP1 axis in tumorigenesis and hormonal therapy resistance

TASK1: To determine the biological significance of CDK2 mediated phosphorylation of PELP1

Generation of PELP1-CDK mutant plasmid constructs and model cells: I have found that

CDK2/CycE and CDK2/CycA2 both phosphorylate PELP1 (Fig1A) and utilizing various deletion mutants (Fig. 1B) and site directed mutagenesis of putative CDK2 phosphorylation sites on PELP1, I have identified Ser 991 and Ser 477 as the CDK2 phosphorylation sites (Fig. 1C). To further study the *in vivo* significance of CDK phosphorylation of PELP1, an N-terminal GFP-tagged PELP1 mutant that lacked these two CDK sites were generated using site directed mutagenesis (Ser to Ala mutant) (Fig. 1D). ZR75 cells stably expressing PELP1-WT and PELP1-MT (single and pooled clones) were generated. ZR75 cells were chosen based on our earlier findings that ZR cells express relatively low levels of endogenous PELP1, and thus were more suitable to study the functions of exogenously expressed PELP1 than other ER-positive cells such as MCF7. In general, these stable clones express 3- to 4-fold more PELP1-MT than endogenous PELP1, and mutant expression is equivalent to GFP-tagged PELP1-WT clone and migrated to the expected size on SDS-PAGE when detected using the GFP antibody (Fig. 1E). Both PELP1-WT and PELP1-MT localized to the nuclear compartment when detected employing immunofluorescence (Fig.1F). Phosphorylation mimetic constructs (Ser to Glu mutant) has been constructed and confirmed by sequencing.

Effect of CDK2 phosphorylation site mutations in PELP1 on E2 mediated cell cycle progression. To test if the CDK2 phosphorylation contributed to PELP1's ability to drive cell cycle progression, I first compared the cell proliferation rate between PELP1-WT and PELP1-MT expressing model cell lines under 10% serum conditions using Cell Titer-Glo Assay. Asynchronized cells (ZR-75 controls, PELP1-WT, PELP1-MT) were plated equally in a 96 well clear bottom plate

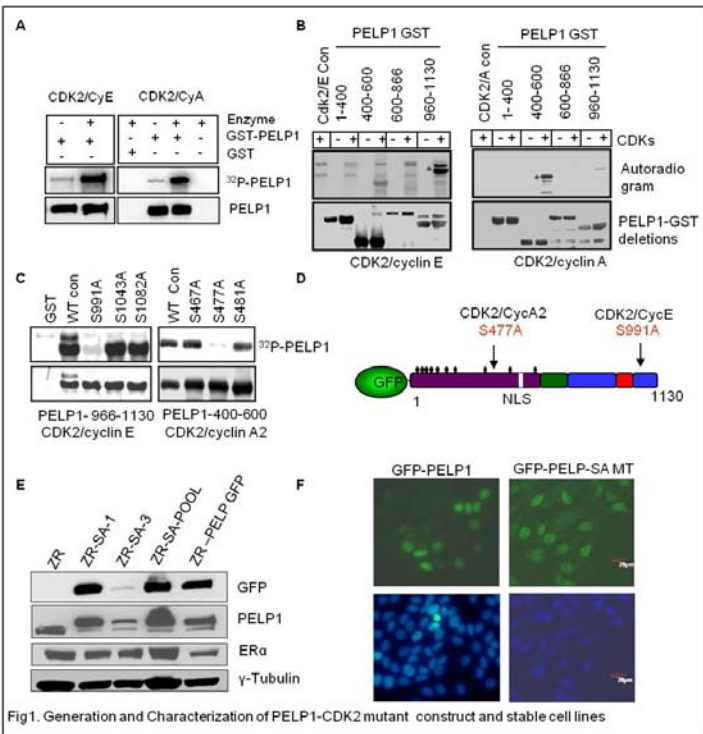


Fig1. Generation and Characterization of PELP1-CDK2 mutant construct and stable cell lines

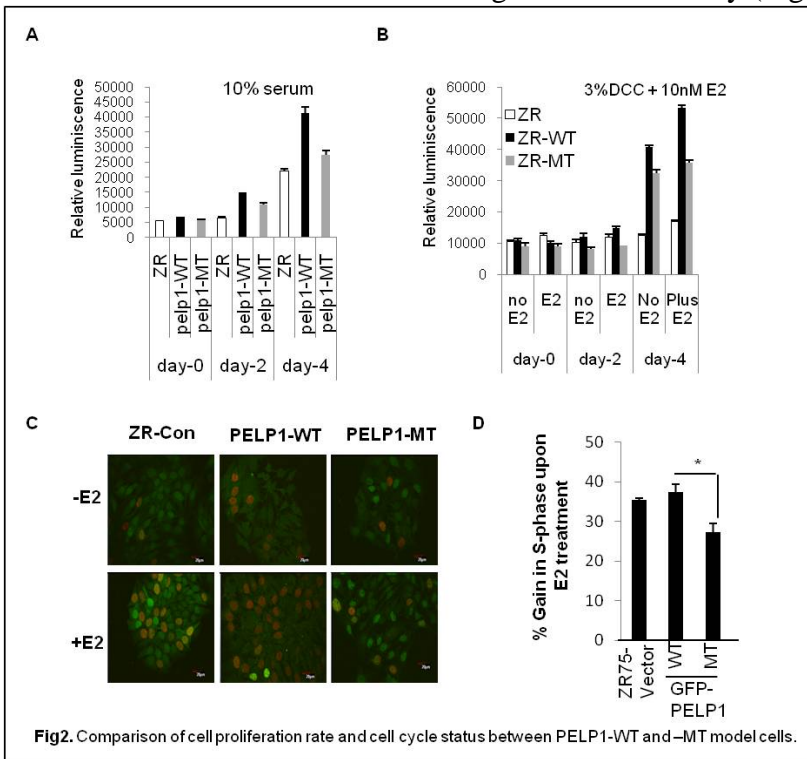


Fig2. Comparison of cell proliferation rate and cell cycle status between PELP1-WT and -MT model cells.

and after 48 and 96 h, rate of proliferation was assayed following manufacturer's instructions. As expected from the previous studies, PELP1-WT expression increased cell proliferation compared ZR75 control cells. CDK2 site mutants clearly lagged behind the PELP1-WT cells (Fig. 2A). Furthermore, to assess how the mutant cells respond to estrogen stimulation, I plated ZR-75 control, PELP1-WT and PELP1-MT cells in a 96 well clear bottom plate in 3% dextran charcoal treated serum followed by 100nM estrogen treatment. Cell proliferation assay revealed that proliferation rate in response to E2 in mutant cells was significantly less compared to the PELP1-WT cells (Fig. 2B). This finding was corroborated by results obtained from our BrdU labeling assay. ZR75 stably expressing GFP or GFP tagged PELP1-WT or GFP tagged PELP1-MT were synchronized to G₀ phase by serum starvation, cells were labeled with BrdU and released into cell cycle by addition of 10% FBS containing serum for 16 h. Results show that PELP1-WT cells acquire more BrdU into DNA depicting increased cells in S phase of cell cycle while PELP1-MT clearly show less cells with BrdU labeling (Fig. 2C). I further analyzed the cell cycle progression of the PELP1-WT and -MT clones by flow cytometry and found that PELP1-WT expression contributed to increased G1-S progression, while mutation of the CDK sites in PELP1 diminished its ability to a great extent (Fig. 2D). Overall, these results suggest that CDK2 mediated phosphorylation is important for PELP1 mediated cell cycle progression.

Mutations in CDK2 phosphorylation sites affect PELP1 functions: I used a knockdown/replacement

strategy to validate the effect of CDK phosphorylation on PELP1-mediated proliferation. ZR75 cells that stably express PELP1 shRNA were transfected with shRNA-resistant PELP1-WT or -MT expression vectors using nucleofactor protocol (Amaxa) that achieved >90% transfection efficiency (Fig.3A). After 48 h, transfected cells were allowed to proliferate with or without E2. Results showed knock-down of PELP1 in ZR75

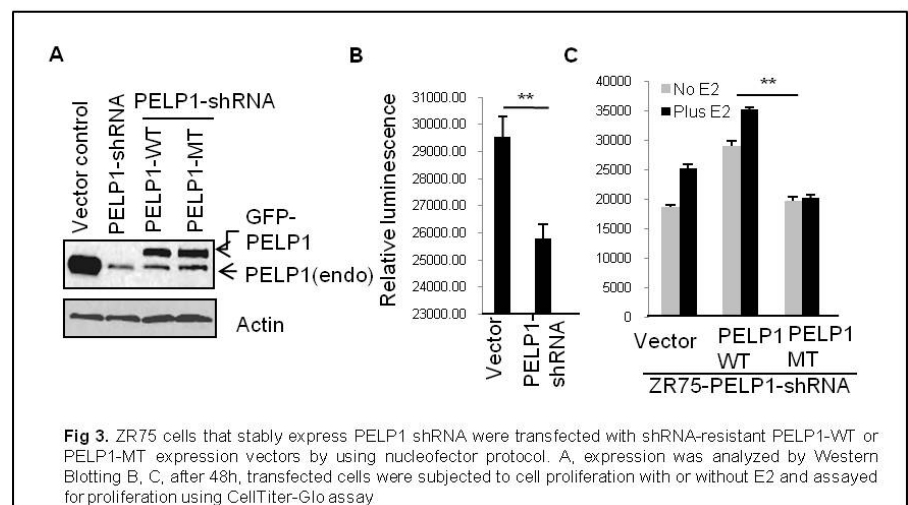


Fig 3. ZR75 cells that stably express PELP1 shRNA were transfected with shRNA-resistant PELP1-WT or PELP1-MT expression vectors by using nucleofactor protocol. A, expression was analyzed by Western Blotting B, C, after 48h, transfected cells were subjected to cell proliferation with or without E2 and assayed for proliferation using CellTiter-Glo assay

by PELP1-shRNA reduced cell proliferation (Fig. 3B) and replacement of shRNA resistant PELP1-WT but not PELP1-MT enhanced E2-mediated proliferation (Fig.3C). These findings suggest that phosphorylation by CDKs is biologically relevant for PELP1-mediated G1-S phase transition.

Generation and characterization of phosphorylation-state-specific-antibodies for PELP1. To further characterize the *in vivo* relevance of the identified sites, I made an attempt to generate rabbit polyclonal phospho-specific antibodies against each of phospho-S991 and phospho-S477 sites using commercial facility (Openbiosystems/Fischer Sci). The peptide sequence used for generating phospho-antibody against S477 and S991 were SPPADALKLR(pS)PRGSPDGSLQ and TLPPALPPPE(pS)PPKVQPEPEP respectively. I was only successful in obtaining S991-phospho-antibody, while failed to generate S477-phospho-antibody; probably because of the poor antigenicity of the S477 peptide. The PELP1 S991 antibody was affinity purified using positive absorption with phospho-peptide followed by negative adsorption with unmodified peptide. The purified antibody titer was quantitated by indirect-ELISA (Data not shown). The antibody efficiently recognized phosphorylated endogenous and GFP-tagged PELP1, but was unable to detect the Ser991 to Ala PELP1

mutant (Fig. 4A). Ser991 phosphorylation was found to be increased when G0/G1 synchronized cells were treated with estrogen for various time periods (Fig. 4B). Furthermore, the phosphorylation showed a gradual decrease when cells were released into cell cycle from double thymidine arrested cells into other phases of the cell cycle by addition of thymidine free medium (Fig. 4B). Similarly, normal transformed IMR-90 and NIH3T3 were synchronized to G1 phase by serum starvation and released into the cell cycle by addition of 10% serum and the expression of PELP1 was analyzed on a 4-12% Bis-Tris gradient gel. I found that human fibroblast cells exhibited two different mobility shifts in PELP1; a slow-migrating form and a fast-migrating form (Fig. 4C, left panel) while in murine fibroblast cells there were two slower moving forms during various phases of cell cycle (Fig. 4C, right panel). To examine whether the PELP1 Ser991 antibody recognized the shifted bands seen in normal IMR-90 cells, I synchronized IMR-90 cells to G1 phase and released them into the cell cycle. Analysis of cell lysates on a 4-12% gradient gel revealed that the phospho PELP1 antibody recognized both forms (Fig. 4D). Recognition of the two forms by PELP1 phospho-specific antibody suggests that PELP1 gets phosphorylated at multiple residues upon cell cycle progression (hypo and hyper-phosphorylated states) similar to another cell cycle regulatory protein pRb. Overall these results demonstrate that CDK2 can phosphorylate PELP1 *in vivo*.

The *in vivo* kinetics of PELP1 phosphorylation: To validate whether PELP1 is phosphorylated *in vivo*, I synchronized MCF7 cells to G₀/G₁ by serum starvation, labeled the cells with ³²P-orthophosphate and treated the cells with E2 for different periods of time. I then performed immunoprecipitation of PELP1 using endogenous PELP1 antibody and found that PELP1 exists as a phospho-protein with two peaks of phosphorylation (early 2-5 h and later 10-13 h) after E2 stimulation (Fig. 5A). To confirm the CDK2 phosphorylation sites in PELP1, 293T cells were co-transfected with either PELP1-WT or PELP1-MT construct with or without Cyclin E followed by ortho-phosphate labeling and Immunoprecipitation using anti-GFP antibody. Auto-radiograph confirmed that

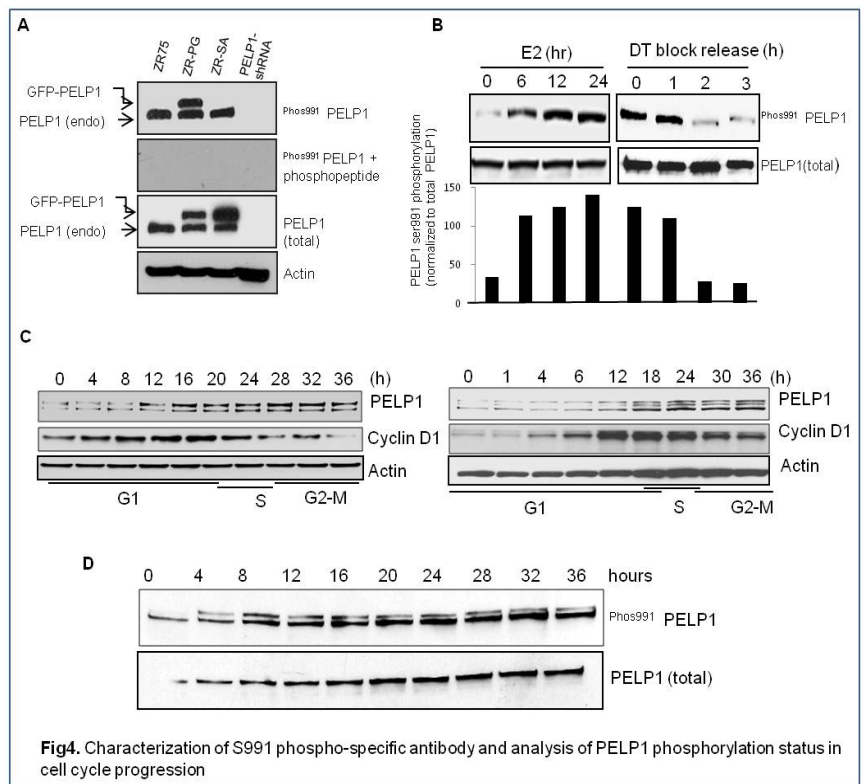


Fig4. Characterization of S991 phospho-specific antibody and analysis of PELP1 phosphorylation status in cell cycle progression

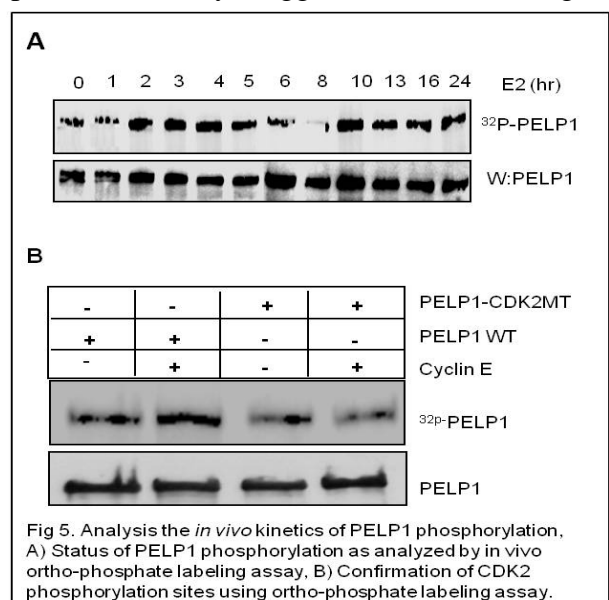


Fig 5. Analysis the *in vivo* kinetics of PELP1 phosphorylation. A) Status of PELP1 phosphorylation as analyzed by *in vivo* ortho-phosphate labeling assay, B) Confirmation of CDK2 phosphorylation sites using ortho-phosphate labeling assay.

the CDK2 site mutations in the context of full-length PELP1 significantly reduce CDK2/CycE-mediated PELP1 phosphorylation (Fig. 5B).

Effect of CDK2 phosphorylation on PELP1 mediated E2F transactivation function: Since PELP1 is a pRb binding protein and CDKs phosphorylate PELP1, I investigated whether CDKs phosphorylation of PELP1 aids in E2F functions. First, I compared the expression of genes involved in cell cycle progression between MCF7 and MCF7-PELP1-shRNA stable cells using a focused microarray approach. Commercially available Oligo GEArray® Human Cell Cycle Microarray (SABiosciences, Frederick, MD) that contains 112 genes involved in cell cycle regulation was used and target genes whose expression was differentially regulated (at least 2 fold difference) upon PELP1 depletion were identified.

Down regulation of PELP1 substantially reduced the expression of a number of cell cycle genes including Cyclin D1, pRb, cyclin B2 and CDC25C (Fig. 6A). I then examined whether PELP1 enhances E2F-mediated gene activation and whether CDK phosphorylation affects PELP1 activation of E2F functions using E2F luciferase reporter. PELP1 knock down substantially reduced the E2F reporter gene activity (Fig. 6B, left panel). The cells with PELP1-WT over expression had greater E2F luciferase reporter activity, while PELP1-MT that lacked CDK phosphorylation sites failed to enhance the E2F reporter activity (Fig. 6B, right panel). Chromatin immune-precipitation demonstrated that PELP1 is recruited to the promoters of the E2F target gene promoters Cyclin A and Cyclin E that contain E2F binding sites (Fig. 6C). However, mutation of CDK phosphorylation sites in PELP1 did not affected its recruitment over E2F target genes (data not shown). Further, qRT-PCR also demonstrated that many of the E2F target genes are down-regulated upon transient PELP1-siRNA treatment in MCF7 or stable knockdown of PELP1 in ZR75-1 cells (Fig. 6D). The effect of PELP1 CDK2 mutants on ERE luciferase reporter activity will be studied in the final year of study.

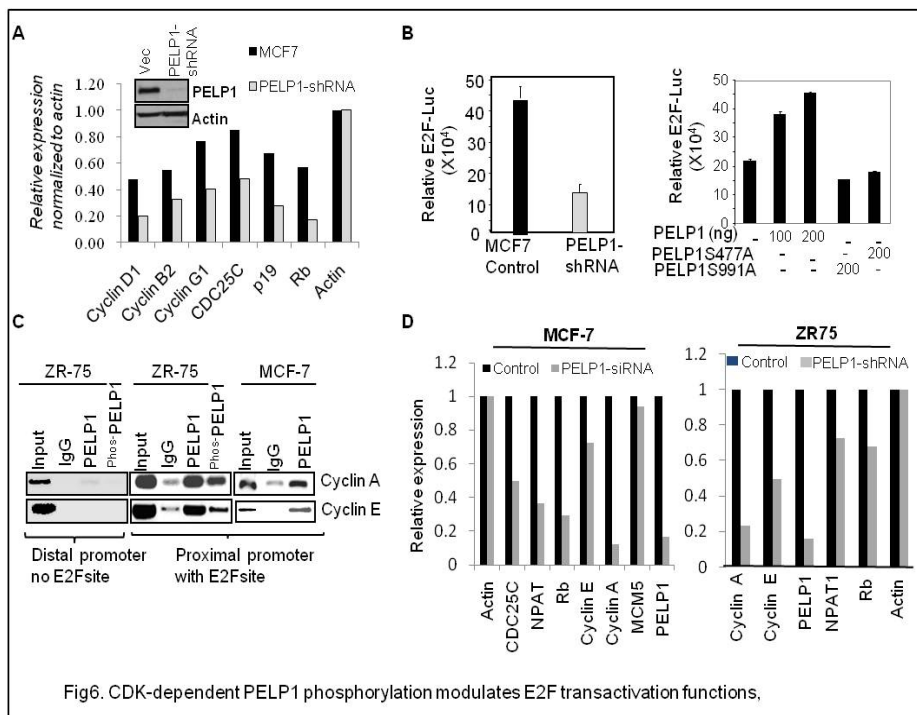
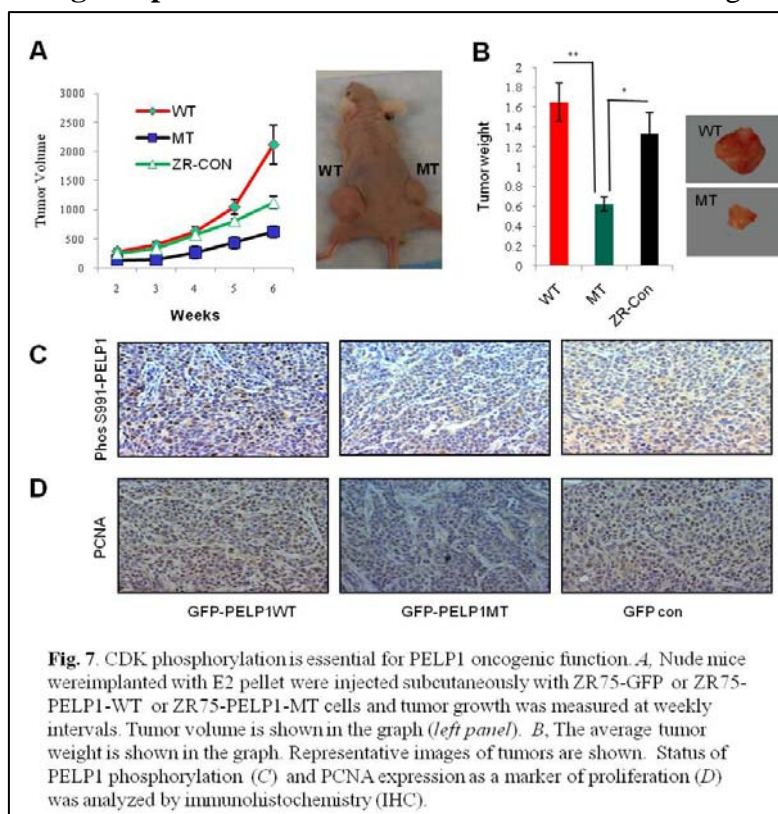


Fig6. CDK-dependent PELP1 phosphorylation modulates E2F transactivation functions,

Task3. To investigate the therapeutic potential of CDK-PELP1 axis in tumorigenesis and hormonal therapy resistance.

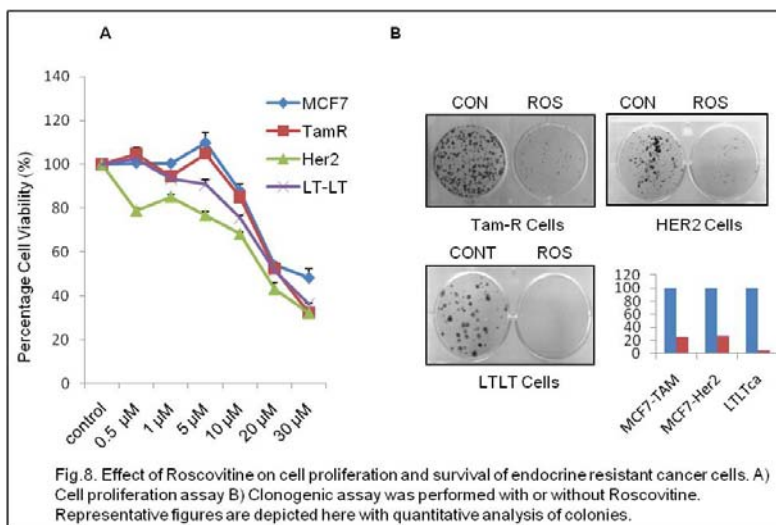
Mutation of CDK sites decreases PELP1 oncogenic potential in vivo: I used a nude mouse xenograft

model to examine whether CDK phosphorylation of PELP1 is required for tumorigenic potential of breast cancer cells *in vivo* using model cells that express either PELP1 or PELP1-MT that lack CDK phosphorylation sites. Both vector-transfected and PELP1 WT-expressing cells formed tumors and these tumors grew linearly with time (Fig. 7A). However, PELP1-MT injected sites had smaller tumors than those that developed in the controls (Fig. 7B). Compared to both mutant and parental ZR75 cells, ZR-PELP1-WT cells had more PELP1 phosphorylation at Ser991 (Fig. 7C) PCNA staining of the tumor sections revealed greater proliferation in the PELP1-WT xenograft tumors than in the PELP1-MT tumors (Fig. 7D). These results suggested that CDK phosphorylation of PELP1 is essential for optimal growth of E2-driven tumor growth *in vivo*.



Blocking CDK2-PELP1 axis using Roscovitine suppresses proliferation and survival of therapy resistant cells:

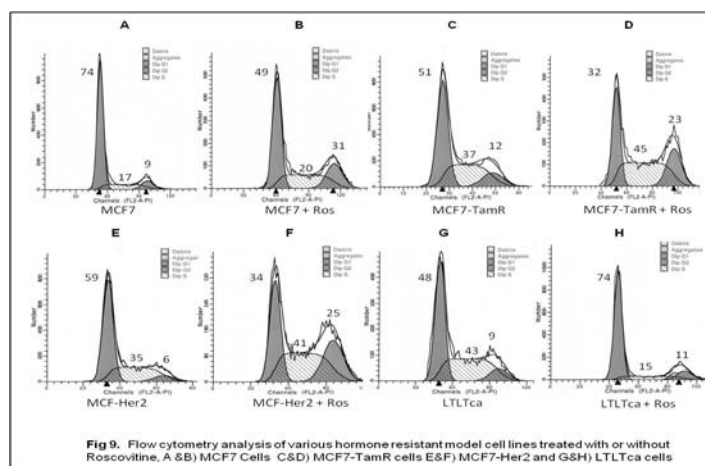
Based on earlier studies that show enhanced CDK2 activation and PELP1 expression in hormone therapy resistant breast cancer cells, I hypothesized that blocking CDK2-PELP1 axis will have therapeutic benefits. To address this hypothesis, I employed three different model cell lines that exhibit hormonal therapy resistance, MCF7-Her2, MCF7-Tam and LTLTca and treated them with or without a potent CDK2 inhibitor, Roscovitine that is currently in phase II trial for non small cell lung cancer (NSCLC) and nasopharyngeal cancer (NPC). The cell proliferation was measured using a luminescence based cell proliferation assay (Cell Titer Glo). As a positive control, MCF7 cells showed a dose dependent decrease in cell proliferation. All the three endocrine resistant cells, MCF7-Her2, MCF7-Tam and LTLTca showed concomitant decrease in cell proliferation upon increasing Roscovitine concentration (Fig. 8A). All the three cell lines showed 50% or more reduction in cell proliferation (IC_{50}) as compared to the untreated control group at a dose range of 20-30 μ M.



LTLTca, MCF7-Her2 and MCF7-TamR cells were more sensitive to 30 μ M Roscovitine as compared to parental MCF7 cells. In addition to proliferation assay, clonogenic survival of endocrine resistant cells was also assessed using an *in vitro* clonogenic survival assay. All the model cells that were treated with 20 μ M of Roscovitine for 7 days, showed a dramatic reduction in their clonogenic ability (Fig. 8B). Both MCF7-Tam and MCF7-Her2 cells showed ~75% reduction in colony formation potential. When compared to other model cells, LTLTca upon treatment with Roscovitine showed on 4% colony formation as compared to untreated cells. Overall the results suggest that blocking CDK2 activity using Roscovitine has the potential to suppress the proliferation and survival potential of endocrine resistant cells at a dose range of 20-30 μ M.

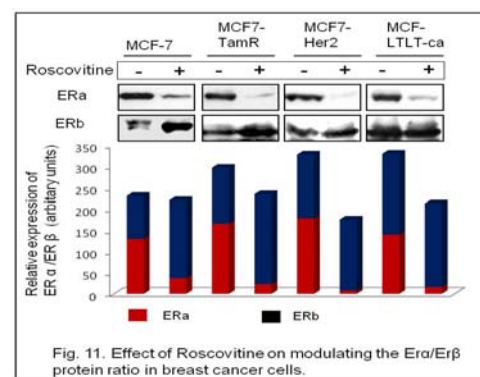
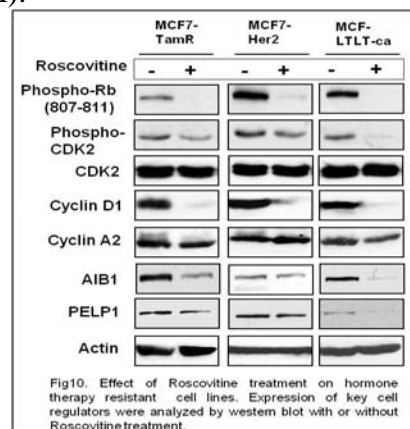
Roscovitine promotes cell cycle arrest of endocrine therapy resistant cells:

Previous studies have shown that Roscovitine has the potential to perturb the cell cycle progression in various cell lines [42]. To evaluate whether Roscovitine induces cell cycle arrest in endocrine therapy resistant cells, I treated different model cells with 20 μ M Roscovitine for 24 h. With the exception of LTLTca cells, Roscovitine treated MCF7, MCF7-Tam, MCF7-Her2 showed a substantial increase in cells containing 4N DNA content (G2/M phase) with concurrent decrease in cells in G1 phase (Fig. 9 A-F). Unlike other model cells, 74% of Roscovitine treated LTLTca cells accumulated in G1 phase as compared to 48% G1 phase cells in untreated LTLTca cells (Fig.9 G&H).



Roscovitine treatment promotes down regulation of key cell cycle regulators in therapy resistant cells.

Since Roscovitine is a potent CDK2 inhibitor and induces cell cycle arrest in endocrine resistant cells, I tested the expression of key cell cycle regulators upon Roscovitine treatment. First of all, I confirmed that Roscovitine indeed inactivate CDK2 by assessing the Threonine 160 phosphorylation of CDK2. Results show that all model cells show a decrease in phospho-CDK2 (T160) when treated with 20 μ M of Roscovitine for 24 h (Fig. 10) confirming the functional blockage of CDK2 activity. I also tested the level of phospho-Rb, a well known



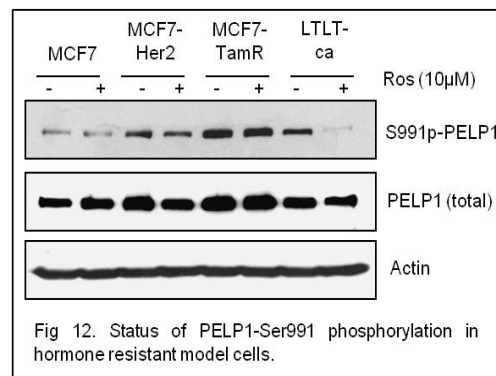
substrate of CDK2 as a marker of decreased CDK2 activity and results suggest that phosphorylation of Rb is completely abolished upon Roscovitine treatment in all the three model cell lines. Furthermore, expression of cellular Cyclin D1 shows a drastic reduction while Cyclin A2 only showed only a moderate reduction upon drug treatment (Fig. 10).

Roscovitine treatment modulate ERα/β ratio in breast cancer cells: Therapy sensitive and resistant model cells were treated with 20 μ M of Roscovitine for 24h and subsequently analyzed the expression of steroid receptor ER alpha and beta. Results indicate

ERα/β ratio in breast cancer cells. Results indicate

that Roscovitine down regulated ER α expression while there is a moderate to no decrease in the ER β expression (Fig. 11 *upper panel*). Quantitative densitometric evaluation of results suggests that there is a possible alteration in ER α / β ratio upon Roscovitine treatment (Fig. 11 *lower panel*).

Hormone therapy resistant cells exhibit enhanced phosphorylation of ER coregulator PELP1: To examine whether CDK influences the functions of ER coregulator via phosphorylation, I examined the phosphorylation status of PELP1 using western analysis. Therapy resistant MCF7-Her2, MCF7-TamR and LTLTca showed increased PELP1 phosphorylation at Ser991 and this phosphorylation is sensitive to Roscovitine treatment (Fig. 12).



III. KEY RESEARCH AND TRAINING ACCOMPLISHMENTS

A. Key Research Accomplishments:

- Establishment of breast model cells model cells with functional and CDK2-defective PELP1 signaling axis and demonstrating that CDK mediated PELP1 phosphorylation is needed for optimal estrogen mediated G1-S cell cycle progression and tumorigenesis (Results are published in Cancer Research journal)
- Establishment of PELP1 as a novel coregulator of E2F and demonstration of the significance of PELP1 in E2F signaling.
- Generation and characterization of a novel phospho-specific PELP1 antibody that uniquely identify CDK phosphorylated PELP1
- Demonstration that CDK2 mediated PELP1 phosphorylation is enhanced in various hormone resistant cell lines.
- *In vitro* demonstration that blocking CDK2 activity in the context of hormone therapy resistance has therapeutic significance.
- Demonstration that Roscovitine (a CDK2 inhibitor) has the potential to modulate the ER alpha/beta ratio in breast cancer cells.

B. Key Training Accomplishments during last year:

- **Journal Club Attendance and Presentations:** I have attended “Hormones and Cancer Journal Club” organized bi-weekly at Dept of OB-GYN and presented three critical papers from high impact journals during last one year.
- **Department Seminar Series and Lab meetings:** I have attended all the mandatory Seminars and invited speaker presentations organized at Dept of Molecular Medicine. In addition to that, I have participated in all lab meetings organized in Dr Vadlamudi lab on a weekly basis.
- **Oral Presentations:** Have presented my data during Annual Departmental Retreat, held on Sept 2010 (Dept of Molecular Medicine).

- **Teaching and Supervising:** Have completed all my teaching assistant duties (2008-09) at Dept of Molecular Medicine. I also had the opportunity to supervise an MS student on his research project (Yr-2010).
- **Conferences/Symposiums:** I have attended the Annual AACR conference-2010 and SABCS (San Antonio Breast Cancer) symposium-2010 and presented research posters.
- **Honors and Awards:** In 2010, I received '**The Overall Grand Prize**' for the oral presentation at Annual Departmental Retreat held at Dept of Molecular Medicine (2010) and **First Prize for Research poster presentation** at CTIRC-Cancer Development & Progression Program Retreat (2010), held at San Antonio, TX

IV. REPORTABLE OUTCOMES

A. Overall Publications:

1. **Nair B.C**, Nair S.S, Chakravarty D, Challa R, Manavathi B, Yew P.R, Kumar R, Tekmal R.R, Vadlamudi R.K (2010). CDK-mediated phosphorylation plays a critical role in PELP1's oncogenic functions. *Cancer Res.* **70(18): 7166-75.**
2. Cortez V, **Nair BC**, Chakravarty D, Vadlamudi RK.(2011) Integrin-linked kinase 1: role in hormonal cancer progression. *Front Biosci.*, **1(3): 788-96.**
3. **Nair BC** and Vadlamudi RK. (2010). ZD6474 coerces breast cancer for an apoptotic journey. *Cancer Biology and Therapy* **9(8):1-3.**
4. **Nair BC** and Vadlamudi RK (2008). Regulation of hormonal therapy Resistance by cell cycle machinery. *Gene Therapy and Molecular Biology* **12:395-404.**

B. Poster presented in 2010:

1. **Nair BC**, Vallabhaneni S, Li R, Tekmal RR, Vadlamudi RK (2010). Roscovitine confers tumor suppressive effect on therapy-resistant breast tumor cells. *Presented at 33rd Annual San Antonio Breast Cancer Symposium.*
2. **Nair BC**, Morales M, Nair HB, Vallabhaneni S, Li R, Tekmal RR, Vadlamudi RK (2010). Roscovitine modulates ER α /ER β ratio: A novel means to target hormonal therapy resistance. *Proceedings of the 101st Annual Meeting of the American Association for Cancer Research; 2010 Apr 17-21; Washington, DC. Philadelphia (PA): AACR; 2010*

C. Oral Presentation in 2010:

1. **Nair BC** and Vadlamudi RK (2010). Roscovitine Confers Tumor Suppressive Effect on Therapy-resistant Breast Cancer Cells. *Symposium on Advances in Cell Signaling, Cancer Prevention and Therapy*, South Padre Island.

V. CONCLUSIONS: Estrogen is known to promote key cell cycle events like activation of CDK and hyper-phosphorylation of pRB in ER-positive breast epithelial cells, leading to increased rate of G1/S

phase transition. *In the first year of grant support* from DOD-BCMRP, I have found that PELP1 is a novel substrate of CDK2 and that its phosphorylation is important for estrogen mediated cell cycle progression using ZR75 model cells that harbors over-expressed dominant PELP1-CDK2 site mutant. Further, I have developed the first ever phospho-antibody against PELP1 to study its function in vivo. *In the second year of grant support*, I used the phospho antibody and established its role in cell cycle progression and tumorigenesis. In addition I have now also established the role of PELP1 phosphorylation in modulating E2F mediated gene transcription and PELP1 mediated tumorigenesis and demonstrated that PELP1 acts as a coregulator of E2F. I have provided strong evidence for the therapeutic significance of blocking CDK2-PELP1 axis in the context of hormone therapy resistance. My studies during second year also demonstrated Roscovitine's ability to modulate ER alpha/beta ratio. *In the next year of funding*, I will continue to examine the role of CDK –PELP1 axis in the progression and for therapeutic utility. I intend to analyze the *in vivo* efficacy of blocking CDK2-PELP1 axis using preclinical xenograft based assays and also evaluate the role of CDK2 mediated phosphorylation of PELP1 on histone biosynthesis. Furthermore, evaluation of phospho-PELP1 expression in breast cancer tumor progression arrays (TMA) will be done in the final year of pre-doctoral grant support.

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VI. APPENDICES

1. **Nair B.C**, Nair S.S, Chakravarty D, Challa R, Manavathi B, Yew P.R, Kumar R, Tekmal R.R, Vadlamudi R.K. **(2010)**. CDK-mediated phosphorylation plays a critical role in PELP1's oncogenic functions. *Cancer Res.* 70(18): 7166-75.

Cyclin-Dependent Kinase–Mediated Phosphorylation Plays a Critical Role in the Oncogenic Functions of PELP1

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Abstract

Estrogen receptor (ER) signaling plays an important role in breast cancer progression, and ER functions are influenced by coregulatory proteins. PELP1 (proline-, glutamic acid-, and leucine-rich protein 1) is a nuclear receptor coregulator that plays an important role in ER signaling. Its expression is deregulated in hormonal cancers. We identified PELP1 as a novel cyclin-dependent kinase (CDK) substrate. Using site-directed mutagenesis and *in vitro* kinase assays, we identified Ser⁴⁷⁷ and Ser⁹⁹¹ of PELP1 as CDK phosphorylation sites. Using the PELP1 Ser⁹⁹¹ phospho-specific antibody, we show that PELP1 is hyperphosphorylated during cell cycle progression. Model cells stably expressing the PELP1 mutant that lack CDK sites had defects in estradiol (E2)–mediated cell cycle progression and significantly affected PELP1-mediated oncogenic functions *in vivo*. Mechanistic studies showed that PELP1 modulates transcription factor E2F1 transactivation functions, that PELP1 is recruited to pRb/E2F target genes, and that PELP1 facilitates ER signaling cross talk with cell cycle machinery. We conclude that PELP1 is a novel substrate of interphase CDKs and that its phosphorylation is important for the proper function of PELP1 in modulating hormone-driven cell cycle progression and also for optimal E2F transactivation function. Because the expression of both PELP1 and CDKs is deregulated in breast tumors, CDK-PELP1 interactions will have implications in breast cancer progression. *Cancer Res*; 70(18); 7166–75. ©2010 AACR.

Introduction

Deregulation of the cell cycle is one of the hallmarks of cancer and is governed by the core cell cycle proteins such as retinoblastoma (pRb) and cyclin-dependent kinases (CDK; ref. 1). Although the role of CDKs in cell cycle progression is well established, defining the complete substrate repertoire of CDKs remains an enigma; many of their potential substrates are yet to be identified. In spite of the known redundancy among CDKs, CDK4 and CDK2 cooperatively play an important role in the G₁-S transition as attested by the mid-gestational embryonic lethality of the *CDK2*^{−/−}/*CDK4*^{−/−} double-knockout mice and a delayed G₁-S transition (2). Recent studies also found that CDK2 and CDK4 are essential for various oncogene-mediated tumorigenesis and that use of

CDK2/CDK4 inhibitors may be a viable option for treatment of tumors with wild-type (WT) p53 (3). Collectively, these emerging studies suggest that phosphorylation of downstream effector proteins by these interphase CDKs plays a crucial event in tumorigenesis.

Estradiol (E2) via the estrogen receptor (ER) promotes cell proliferation in a wide variety of tissues, including mammary glands, and is implicated in breast cancer initiation and progression (4). Estrogen recruits noncycling cells into the cell cycle and promotes G₁ to S cell cycle phase progression. Induction of the early response genes (such as *c-myc* and *c-fos*) is proposed as one mechanism of this process (5–7), whereas regulation of CDK2 and CDK4 activities is proposed as another (8–10). In addition, cyclin D1 was identified as a target of E2 action, and estrogen treatment was shown to upregulate cyclin D1 levels (11). However, the molecular mechanisms underlying E2 regulation of G₁-S phase transition are not completely understood.

PELP1 (proline-, glutamic acid-, and leucine-rich protein 1), a nuclear receptor coregulator, plays an important role in ER signaling (12). PELP1 is a recently discovered proto-oncogene (13) that exhibits aberrant expression in many hormone-related cancers (12) and is a prognostic indicator of shorter breast cancer–specific survival and disease-free intervals when overexpressed (14). PELP1 seems to function as a scaffolding protein with no known enzymatic activity (12), and the mechanism by which PELP1 promotes oncogenesis remains elusive. We have previously shown that PELP1 overexpression promotes E2-mediated G₁-S progression (15).

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Although these findings suggest that PELP1 may play a role in cell cycle progression, little is known about the molecular mechanism(s) responsible for its oncogenic function.

In this study, we identified PELP1 as a novel substrate of CDKs and found that CDK phosphorylation is important for the proper function of PELP1 in modulating hormone-driven cell cycle progression and also for optimal E2F transactivation function. Our findings revealed a novel mechanism by which CDKs use nuclear receptor coregulators to assist cell cycle progression.

Materials and Methods

Cell lines and reagents

Human breast cancer cells MCF7, ZR75, IMR-90, NIH3T3, and 293T were obtained from the American Type Culture Collection. All stable cell lines were generated through 500 μ g/mL G418 (neomycin) selection. E2 was purchased from Sigma. The PELP1 antibody was from Bethyl Laboratories. Recombinant enzyme complexes (CDK4/cyclin D1, CDK2/cyclin E, and CDK2/cyclin A) and CDK antibodies were purchased from Cell Signaling Technology. Anti-green fluorescent protein (GFP) antibody was purchased from Clontech. PELP1 SMARTpool small interfering RNA (siRNA) was purchased from Dharmacon. Phospho-PELP1 antibody was generated by Open Biosystems (Thermo Fisher Scientific) against phospho-PELP1 Ser⁹⁹¹ [peptide sequence TLPPALPPPE(pS) PPKVQPEPEP]. PELP1 220B2 antibody was generated by University of Texas Health Science Center core facility. The plasmids glutathione *S*-transferase (GST)–PELP1 deletions (16), E2F-Luc (17), and GFP-PELP1 (16) were described previously. Expression vectors for *p16INK4A*, *CDK4*, *CDK2*, and *cyclin E* were purchased from Addgene, Inc. Expression vectors for E2F1 and DP1 were purchased from Origene. The PELP1 CDK site mutations were generated on either pGEX-GST-PELP1 deletions or GFP-PELP1 backbone by site-directed mutagenesis (QuikChange Mutagenesis kit, Stratagene). Yeast two-hybrid screening was performed as described (18).

CDK phosphorylation assays

All *in vitro* kinase assays using CDK4 and CDK2 enzymes were performed using the kinase buffer comprising 60 mmol/L HEPES-NaOH (pH 7.5), 3 mmol/L MgCl₂, 3 mmol/L MnCl₂, 3 μ mol/L sodium orthovanadate, 1.2 mmol/L DTT, 10 μ Ci [γ -³²P]ATP, and 100 μ mol/L cold ATP and the purified enzyme complex (100–200 ng/30 μ L reaction). Bacterially or insect cell purified GST-tagged full-length PELP1 and deletions were used as substrates for the *in vitro* CDK kinase assays. Each reaction was carried out for 30 minutes at 30°C and stopped by addition of 10 μ L of 4 \times SDS buffer.

Reporter gene assays

Reporter gene assays were performed by transient transfection using FuGENE6 method (Roche) as described (17). Briefly, cells were transfected using 500 ng of E2F-Luc reporter, 50 ng *E2F*, 50 ng *DP1*, and 10 ng pSV β -galactosidase, with or without 200 ng of PELP1-WT or PELP1-CDK site mutant (MT) expression vectors. Cells were lysed in passive lysis

buffer 36 to 48 hours after transfection, and the luciferase assay was performed using a luciferase assay kit (Promega). Each transfection was carried out in six-well plates in triplicate and normalized with either β -galactosidase activity or the total protein concentration.

Real-time PCR and cell cycle microarray

Cells were harvested with Trizol Reagent (Invitrogen), and total RNA was isolated according to the manufacturer's instructions. cDNA synthesis was done using SuperScript III RT-PCR kit (Invitrogen). Real-time PCR was done using a Cepheid SmartCycler II with specific real-time PCR primers for the E2F target gene (Supplementary Table S1). Results were normalized to actin transcript levels, and the difference in fold expression was calculated using the $\Delta\Delta C_T$ method. Cell cycle microarray was purchased from SABiosciences, and analysis was performed as per the manufacturer's instructions.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) analysis was performed as described previously (19). MCF7 or ZR75 cells expressing GFP-PELP1 WT or MT were cross-linked using formaldehyde, and the chromatin was subjected to immunoprecipitation using the indicated antibodies. Isotype-specific IgG was used as a control. DNA was resuspended in 50 μ L of Tris-EDTA buffer and used for PCR amplification using the specific primers (Supplementary Table S1).

Cell cycle analysis, cell synchronization, and cell proliferation assays

IMR-90 and NIH3T3 cells were synchronized to G₀-G₁ phase by serum deprivation for 3 days and released into the cell cycle by addition of 10% fetal bovine serum-containing medium. MCF7, ZR75, and other derived model cell lines were synchronized to G₀-G₁ phase by serum starvation for 3 days in 0.5% dextran-coated charcoal-treated serum-containing medium and released into the cell cycle by addition of 10⁻⁸ mol/L E2. Double-thymidine block was done to arrest model cells at late G₁ phase (20). Flow cytometry was performed to analyze the cell cycle progression as described previously (15). Cell proliferation rate was measured by using a 96-well format with CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's instructions.

Immunofluorescence, confocal microscopy, and immunohistochemical studies

Cellular localization of PELP1-WT or PELP1-MT was determined by indirect immunofluorescence as described previously (19). Immunohistochemistry was performed using a method as described (21).

Tumorigenesis assays

For tumorigenesis studies, model cells (5 \times 10⁶) were implanted s.c. into the flanks of 6- to 7-week-old female nude mice (*n* = 6 per group) as described (22). Each mice received one 60-day release E2 pellet containing 0.72 mg E2

(Innovative Research of America) 2 days before implantation of cells, and tumors were allowed to grow for 6 weeks. Tumor volumes were measured with a caliper at weekly intervals. After 6 weeks, the mice were euthanized, and the tumors were removed, weighed, and processed for immunohistochemical staining. Tumor volume was calculated using a modified ellipsoidal formula: tumor volume = $1/2 (L \times W^2)$, where L is the longitudinal diameter and W is the transverse diameter (23, 24).

Results

PELP1 is a novel substrate of interphase CDKs

To examine the significance of PELP1 in cell cycle progression, we first analyzed the expression profile of PELP1 throughout the cell cycle using two normal cell lines, IMR-90 (human diploid fibroblast cell line) and NIH3T3 (murine fibroblast cell line), using an antibody (220B2) generated in our laboratory (Supplementary Fig. S1). Serum-starved fibroblast cells were synchronized to G₁ phase and released into the cell cycle by addition of 10% serum, and the expression of PELP1 was analyzed on a 4% to 12% Bis-Tris gradient gel. In human fibroblast cells, two forms of PELP1 were detected: a slow-migrating form and a fast-migrating form (Fig. 1A, left). In murine fibroblast cells, two slower-moving forms and one faster-moving form were present during various phases of the cell cycle (Fig. 1A, right). The PELP1 slower-migrating bands were also detected by the PELP1 antibody that was previously generated in our lab (15) and was raised against NH₂-terminal epitope amino acids 540 to 560 (Fig. 1A). Intriguingly, these slow-migrating bands were difficult to separate on nongradient gels and escaped detection using existing commercial antibodies (data not shown). Shifted PELP1 bands were also seen in various cancer cell lines when stimulated with 10% serum (Supplementary Fig. S1B). Treatment of the total lysates with λ-phosphatase abolished the slower-migrating forms (Supplementary Fig. S1C). These results suggested that PELP1 may be subjected to posttranslational modification, which may be phosphorylation, during cell cycle progression.

To identify potential kinases that phosphorylate PELP1 during cell cycle progression, we performed a yeast two-hybrid screen using mammary gland cDNA library with PELP1 as bait. One of the proteins identified using this screen was CDK4. We confirmed this interaction with purified plasmids in a yeast-based growth assay. The data indicate that the NH₂ terminus (1–400 amino acids) of PELP1 functions as the CDK4-binding region (Supplementary Fig. S2A). Because PELP1 can participate in E2-mediated cell cycle progression, we used breast cancer model cells to further characterize the PELP1-CDK4 interaction. Using coimmunoprecipitation with PELP1 antibody, we found that PELP1 binds CDK4 *in vivo* (Fig. 1B, left) and E2 stimulation enhances the PELP1 interaction with CDK4 (Fig. 1B, middle). In a GST pull-down assay, GST-CDK4 efficiently interacted with the NH₂ terminus of PELP1, and deletion analysis revealed that 200 to 350 amino acids in PELP1 serve as a docking site for CDK4 (Supplementary Fig. S2B). Using *in vitro*

kinase assays with commercially procured CDK4/cyclin D1 and purified GST-tagged PELP1, we found that PELP1 can be efficiently phosphorylated by CDK4 *in vitro* (Fig. 1B, right). Studies have previously shown that E2-mediated cell cycle progression occurs through activation of interphase CDKs (both CDK4 and CDK2; ref. 8). Because the slower-moving forms of PELP1 persisted beyond G₁-S phase (Fig. 1A) and because PELP1 has 11 consensus “Cy” motifs (RXL), which are important for cyclin binding (25, 26), and four CDK2 phosphorylation motifs, we examined whether PELP1 interacts with CDK2 as well. Immunoprecipitation showed that PELP1 interacts with CDK2 (Fig. 1C, left) and that this PELP1-CDK2 interaction occurred at later stages (10–16 hours of E2 stimulation) of cell cycle progression (Fig. 1C, middle). *In vitro* kinase assays using purified CDK2/cyclin E and CDK2/cyclin A complexes further showed that full-length PELP1 may also be a potential substrate of CDK2 (Fig. 1C, right). We further investigated whether CDKs phosphorylate PELP1 *in vivo* by cotransfecting PELP1 with or without CDK4/cyclin D1 and with natural or synthetic inhibitors of CDKs into 293T cells. Cells were metabolically labeled with [³²P]orthophosphoric acid, and PELP1 phosphorylation was measured by using autoradiography after immunoprecipitation. Cotransfection of CDK4/cyclin D1 with PELP1 stimulated phosphorylation of PELP1, whereas cotransfection of the natural CDK4 inhibitor p16INK4 decreased CDK4/cyclin D1-mediated PELP1 phosphorylation (Supplementary Fig. S3A). Both the CDK4 inhibitor Ryuvudine (27) and the CDK2 inhibitor Roscovitine (28) substantially reduced PELP1 phosphorylation (Supplementary Fig. S3B and C), suggesting that PELP1 is a novel substrate of interphase CDKs.

CDKs phosphorylate PELP1 at distinct sites

We next mapped the CDK phosphorylation sites in PELP1 using a deletion and mutagenesis approach. PELP1 was expressed and purified as four small GST fusion domains, which were used as substrates for the *in vitro* kinase assay. CDK4/cyclin D1 phosphorylated PELP1 domains containing 400 to 600 amino acids (Fig. 2A, left), whereas CDK2/cyclin E had a preference toward the PELP1 960–1130-amino acid fragment (Fig. 2A, middle). Interestingly, CDK2/cyclin A2 uniquely phosphorylated PELP1 400 to 600 amino acids but had no activity in the PELP1 960 to 1130 fragment (Fig. 2A, right). We then mutated all the putative consensus CDK sites (S/T/P) and identified specific sites phosphorylated by each of three CDK complexes. We found that CDK4/cyclin D1 preferentially phosphorylated Ser⁴⁷⁷, CDK2/cyclin E phosphorylated Ser⁹⁹¹, and CDK2/cyclin A2 phosphorylated Ser⁴⁷⁷ (Fig. 2B). We also confirmed that these mutations in the context of full-length PELP1 significantly reduce CDK2/cyclin E- and CDK4/cyclin D1-mediated PELP1 phosphorylation when measured by using an *in vivo* orthophosphate labeling assay (Supplementary Fig. S3D).

To further characterize the *in vivo* relevance of the identified sites, we made an attempt to generate rabbit polyclonal phospho-specific antibodies against each of phospho-Ser⁹⁹¹ and phospho-Ser⁴⁷⁷ sites. We were successful only in obtaining

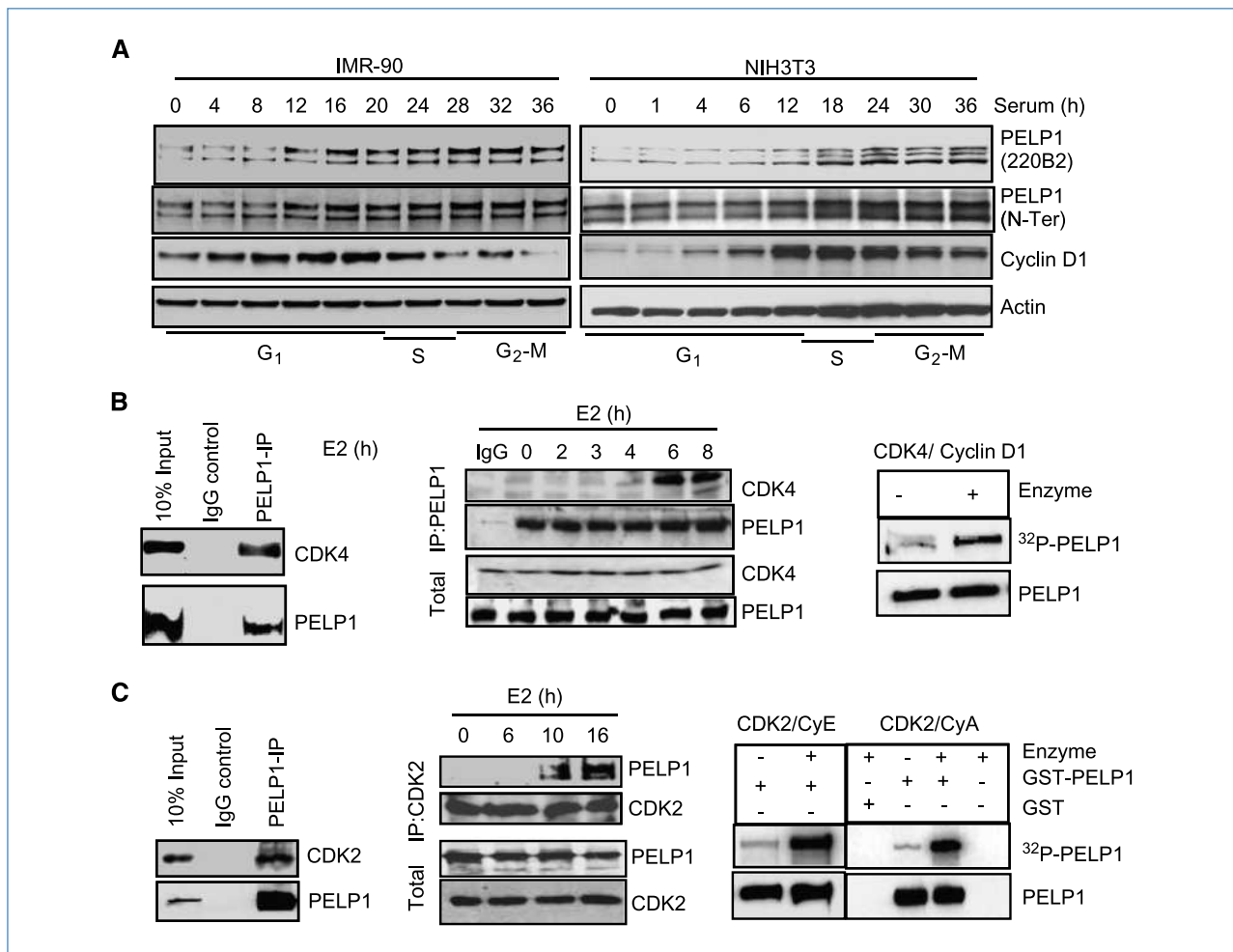


Figure 1. PELP1 is a novel substrate of interphase CDKs. **A**, expression status of PELP1 in the cell cycle was analyzed in IMR-90 cells (left) and NIH3T3 cells (right). Cells synchronized at G₀-G₁ phase were released into the cell cycle, and lysates from different time intervals were used in Western blot analysis with the 220B2 PELP1 antibody. **B**, total lysates from MCF7 cells grown in 10% serum were subjected to immunoprecipitation (IP) using the PELP1 antibody (left), and the CDK4 interaction was verified by Western blotting. Middle, T7-tagged PELP1-overexpressing MCF7 cells were treated with 10⁻⁸ mol/L estrogen for various periods of time, PELP1 was immunoprecipitated, and the CDK4 interaction was verified by Western blotting. Right, *in vitro* kinase assays for the CDK4/cyclin D complex using baculovirus-expressed, GST-tagged, full-length PELP1 as a substrate, and phosphorylation was measured by the amount of ³²P incorporation. **C**, left, total lysates from MCF7 cells grown in 10% serum were subjected to immunoprecipitation using the PELP1 antibody and the CDK2 interaction was verified by Western blot analysis. Middle, MCF7 cells were treated with E2 for various periods of time, and the PELP1 interaction with CDK2 was analyzed by using immunoprecipitation. Right, *in vitro* kinase assays using CDK2/cyclin E (CyE) complex and CDK2/cyclin A2 (CyA) complex using full-length PELP1 as a substrate.

phospho-Ser⁹⁹¹ antibody, whereas we failed to generate phospho-Ser⁴⁷⁷ antibody because of the poor antigenicity of the Ser⁴⁷⁷ peptide. The antibody was specific to PELP1 Ser⁹⁹¹ phosphorylation, as its recognition was efficiently competed by phosphorylated peptide but not by unphosphorylated peptide (Fig. 2C, left). Further, it efficiently recognized phosphorylated endogenous and GFP-tagged PELP1 but was unable to detect the Ser⁹⁹¹ to Ala PELP1-MT (Fig. 2C, left). λ-Phosphatase-treated ZR75 lysates also abolished the ability of the phospho-Ser⁹⁹¹ antibody to recognize phosphorylated PELP1 in these cells (Supplementary Fig. S4A). E2 stimulation substantially increased the level of serine phosphorylation that is recognized by the Ser⁹⁹¹ an-

tibody (Fig. 2D, left), and downregulation of ER signaling by antiestrogen ICI 182780 substantially decreased Ser⁹⁹¹ phosphorylation of PELP1 (Supplementary Fig. S4B). Using double-thymidine block and release of cells, we found that PELP1 Ser⁹⁹¹ phosphorylation accumulated at the G₁-S boundary and gradually decreased thereafter as the cells progressed into other phases of the cell cycle (Fig. 2D, right). PELP1 Ser⁹⁹¹ antibody was able to recognize the shifted bands seen in normal IMR-90 cells in gradient gels when synchronized to G₁ phase and released them into the cell cycle (Supplementary Fig. S4D). Overall, these results show that interphase CDKs can phosphorylate PELP1 at Ser⁹⁹¹ *in vivo*.

Mutations in CDK2 phosphorylation sites affect PELP1 functions

Because previous studies showed that PELP1 augments G₁-S cell cycle phase progression, we investigated the significance of CDK phosphorylation of PELP1 in cell cycle progression. We constructed an NH₂-terminal GFP-tagged PELP1-MT that lacked these two CDK sites. ZR75 cells stably expressing PELP1-WT and PELP1-MT (pooled clones) were

generated. Compared with other ER⁺ breast cancer cells such as MCF7, ZR75 cells express low endogenous PELP1 and therefore represent good model to study the effect of mutations by overexpression. In general, these stable clones express 3- to 4-fold more PELP1-MT than endogenous PELP1, and MT expression is equivalent to GFP-tagged PELP1-WT clone (Fig. 3A). Both PELP1-WT and PELP1-MT are localized in the nuclear compartment in cells and migrated to the

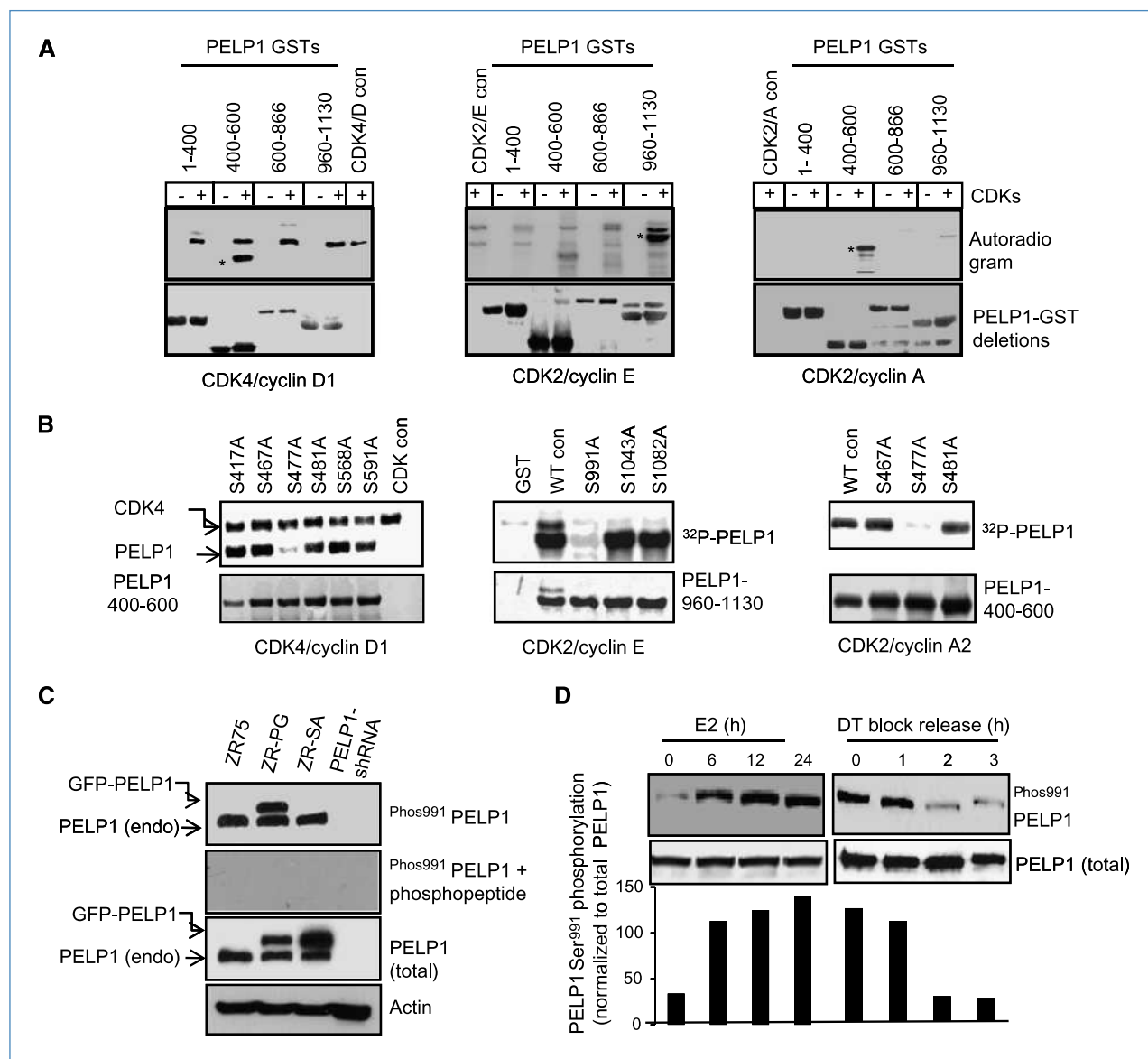


Figure 2. Identification of phosphorylation sites and generation of phospho-specific antibody. A, bacterially expressed GST-PELP1 deletions were used as substrates for an *in vitro* kinase assay using CDK4/cyclin D1 (left), CDK2/cyclin E (middle), and CDK2/cyclin A2 (right), and PELP1 domains phosphorylated by each kinase complex were identified by using autoradiography. B, identification of CDK phosphorylation sites using site-directed mutagenesis (serine to alanine). Various single MTs in the region of interest were used along with respective PELP1-WT deletion fragments. Loss of ³²P incorporation revealed the successful identification of phosphorylation sites. C, Western blot analysis of native, WT-tagged PELP1 and tagged phospho-PELP1-MT with the Ser⁹⁹¹ phospho-PELP1 antibody in the presence or absence of the phosphopeptide. D, MCF7 cells were either arrested and released by E2 stimulation (left) or synchronized into the G₁-S boundary by double-thymidine block and released into the cell cycle by addition of thymidine-free medium (right), and phosphorylation status of PELP1 was analyzed by using the phospho-Ser⁹⁹¹ antibody.

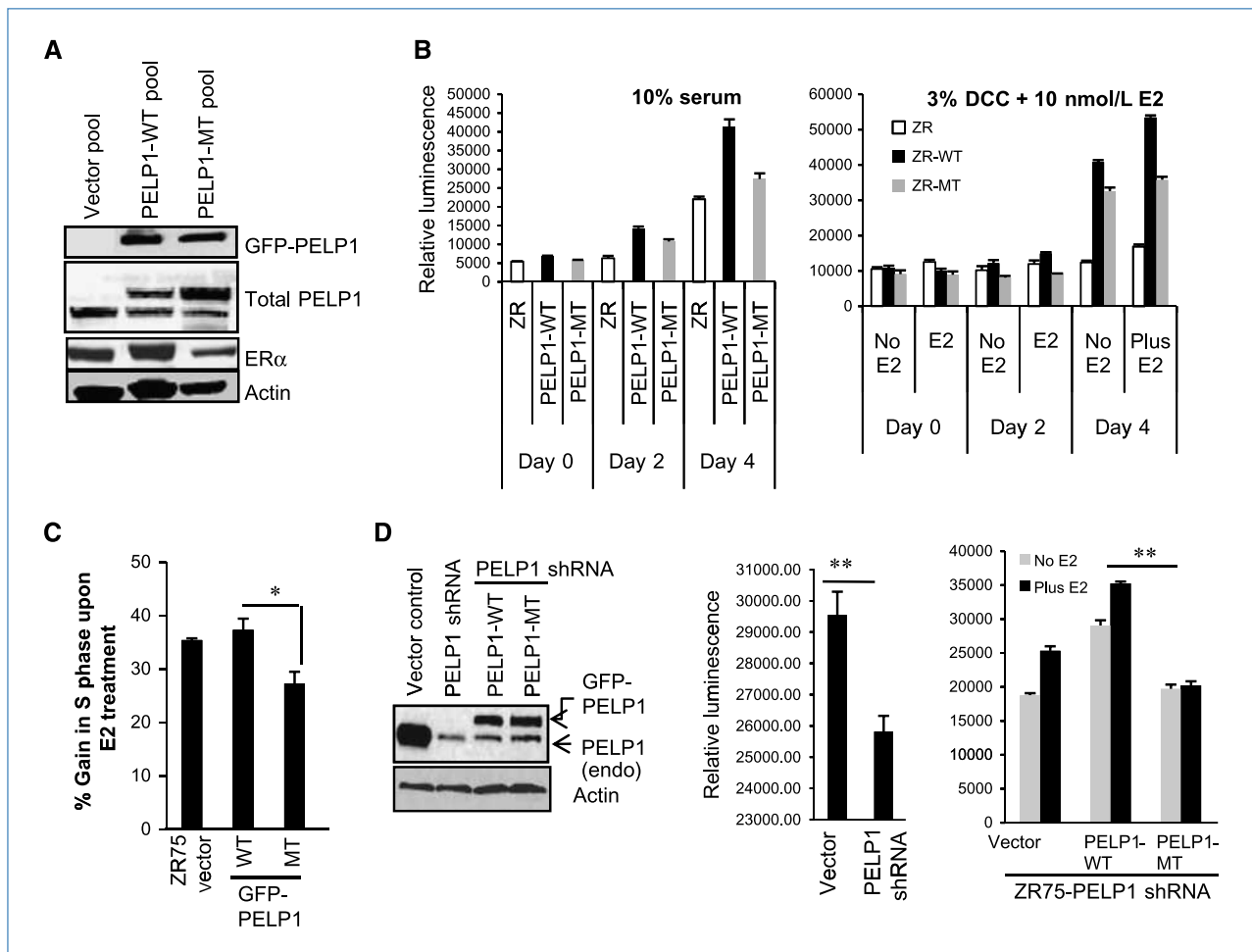


Figure 3. CDK-mediated phosphorylation is required for optimal PELP1 function in cell cycle. A, Western blot analysis of ZR75 cells stably expressing GFP vector, GFP-PELP1-WT, and GFP-PELP1-MT. B, cell proliferation capacity of parental ZR75, PELP1-WT, and PELP1-MT stable cells was analyzed after treating the cells with 10% serum (left) and with or without E2 (right) using CellTiter-Glo assay. C, flow cytometric analysis of cell cycle phases in ZR75-GFP, PELP1-WT, and PELP1-MT pool clones was done and changes observed in S-phase cells are depicted by percentages. D, ZR75 cells that stably express PELP1 shRNA were transfected with shRNA-resistant PELP1-WT or PELP1-MT expression vectors by using nucleofector protocol. After 48 h, transfected cells were subjected to cell proliferation with or without E2 and assayed for proliferation using CellTiter-Glo assay. *, $P < 0.05$; **, $P < 0.001$.

expected size on SDS-PAGE when detected using the GFP antibody (Fig. 3A, left; Supplementary Fig. S5). Western blot analysis using PELP1 phospho-specific antibody showed that GFP-tagged PELP1-WT was phosphorylated during cell cycle progression, whereas PELP1-MT had no signs of phosphorylation (Supplementary Fig. S4C, right). As expected from previous studies, PELP1-WT expression increased cell proliferation as measured by using a CellTiter-Glo assay and bromodeoxyuridine incorporation, whereas mutations in PELP1 diminished its ability to increase cell proliferation (Fig. 3B; Supplementary Fig. S5B). We then analyzed the cell cycle progression of the PELP1-WT and PELP1-MT clones by flow cytometry and found that PELP1-WT expression contributed to increased G_1 -S progression, whereas mutation of the CDK sites in PELP1 diminished the number of cells entering S phase on E2 stimulation (Fig. 3C). We then used a knock-down/replacement strategy to validate the effect of CDK

phosphorylation on PELP1-mediated proliferation. ZR75 cells that stably express PELP1 short hairpin RNA (shRNA) were transfected with shRNA-resistant PELP1-WT or PELP1-MT expression vectors using nucleofector protocol (Amaxa) that achieved >90% transfection efficiency. After 48 hours, transfected cells were allowed to proliferate with or without E2. Results showed that PELP1-WT, but not PELP1-MT, enhanced E2-mediated proliferation (Fig. 3D). These findings suggest that phosphorylation by CDKs is biologically relevant for PELP1-mediated G_1 -S phase transition.

CDK-PELP1 axis modulates expression of E2F target genes

Because PELP1 is a pRb-binding protein and CDKs phosphorylate PELP1, we investigated whether CDK phosphorylation of PELP1 aids E2F functions. First, we compared the expression of genes involved in cell cycle progression

between MCF7 and MCF7-PELP1 shRNA-stable cells using the cell cycle microarray that contains 112 genes involved in cell cycle regulation. Target genes whose expression was differentially regulated (with at least a 2-fold difference) on PELP1 depletion were identified. Downregulation of PELP1 substantially reduced the expression of several cell cycle genes, including *cyclin D1*, *pRb*, *cyclin B2*, and *CDC25C* (Fig. 4A). We then examined whether PELP1 enhances E2F-mediated gene activation and whether CDK phosphorylation affects PELP1 activation of E2F functions by using an E2F-Luc reporter. PELP1 knockdown substantially reduced the E2F reporter gene activity (Fig. 4B, left). The cells with PELP1-WT overexpression had greater E2F-Luc reporter activity compared with vector-transfected cells, whereas PELP1-MT that lacked CDK phosphorylation sites failed to enhance the E2F reporter activity (Fig. 4B, right). ChIP showed that phosphorylated PELP1 is recruited to the promoters of the E2F target gene promoters cyclin A and cyclin E, both of which contain E2F-binding sites (Fig. 4C). However, mutation of CDK phosphorylation sites in PELP1 did not affect its recruitment over E2F target genes (Supplementary

Fig. S5C). However, real-time quantitative PCR analysis showed that PELP1 knockdown significantly decreased the expression of several E2F target genes in both MCF7 and ZR75 cells (Fig. 4D). These results suggest that the phosphorylation of PELP1 by CDKs plays a critical role in the expression of E2F target genes.

Mutation of CDK sites decreases PELP1 oncogenic potential *in vivo*

We used a nude mouse xenograft model to examine whether CDK phosphorylation of PELP1 is required for tumorigenic potential of breast cancer cells *in vivo* using model cells that express either PELP1 or PELP1-MT that lack CDK phosphorylation sites. Both vector-transfected and PELP1-WT-expressing cells formed tumors, and these tumors grew linearly with time (Fig. 5A). However, PELP1-MT-injected sites had smaller tumors than those that developed in the controls (Fig. 5B). Compared with both MT and parental ZR75 cells, ZR75-PELP1-WT cells had more PELP1 phosphorylation at Ser⁹⁹¹ (Fig. 5C). Proliferating cell nuclear antigen (PCNA) staining of the tumor sections revealed greater

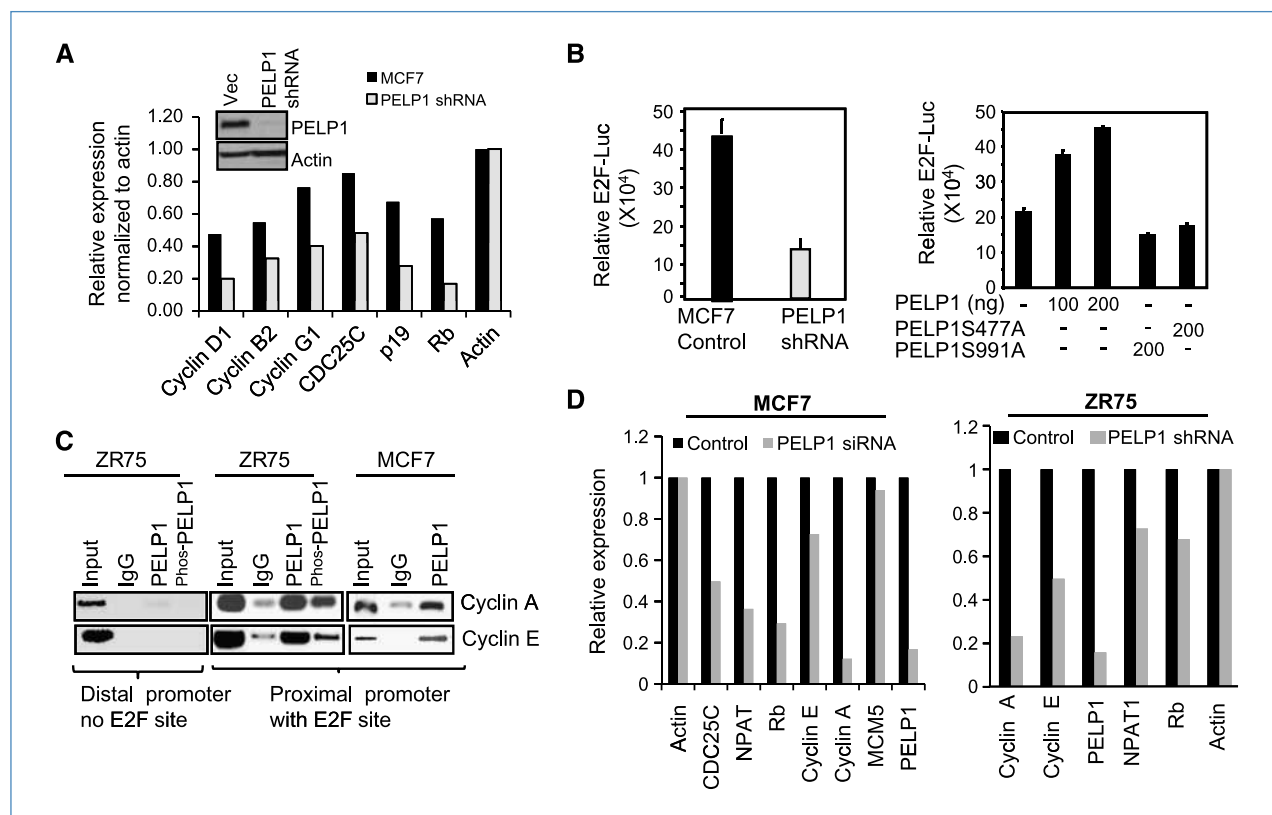


Figure 4. CDK-dependent PELP1 phosphorylation modulates E2F transactivation functions. A, RNA isolated from MCF7 control and stable MCF7-PELP1 shRNA-expressing cells was hybridized to the human cell cycle microarray. Changes in the gene expression were analyzed using SABiosciences software with actin as a control for normalization. Representative genes downregulated on PELP1 depletion are shown. Inset, expression of PELP1 in PELP1 shRNA clones. B, left, MCF7 and MCF7-PELP1 shRNA cells were transfected with E2F-Luc reporter along with E2F and DP1 plasmids, and luciferase activity was measured after 36 h. NIH3T3 cells were transfected with E2F-Luc with E2F and DP1 plasmids and with or without PELP1-WT or PELP1-MT. Right, luciferase activity was measured after 36 h of transfection. C, recruitment of PELP1 to E2F target gene promoter was analyzed by using the ChIP assay with MCF7 and ZR75 cells. D, left, MCF7 cells were transiently transfected with control or PELP1-specific siRNA; right, ZR75 cells were stably transfected with control or PELP1-specific shRNA. Total RNA was isolated and expression of classic E2F target genes was analyzed by real-time quantitative PCR.

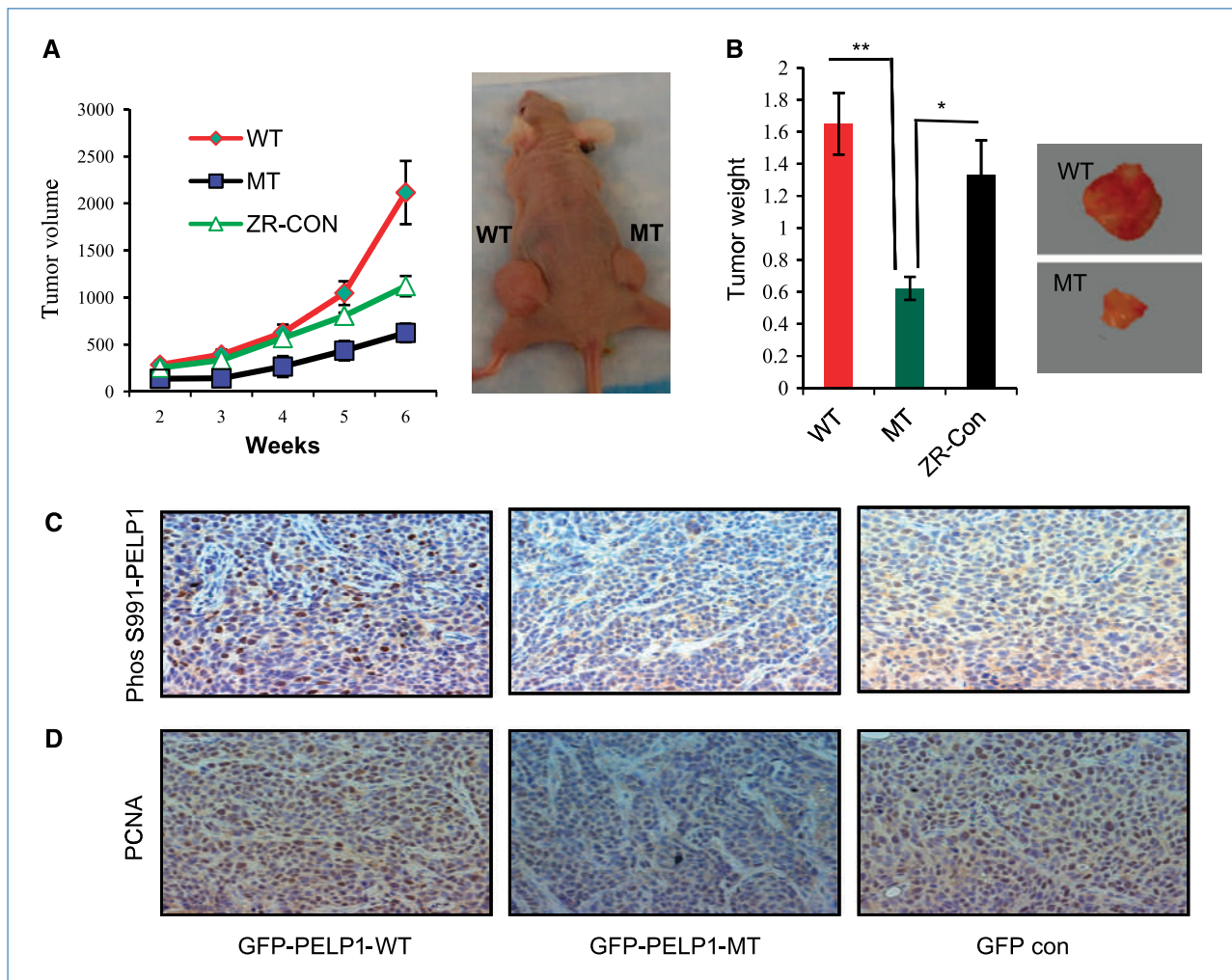


Figure 5. CDK phosphorylation is essential for PELP1 oncogenic function. A, nude mice implanted with E2 pellet were injected s.c. with ZR75-GFP, ZR75-PELP1-WT, or ZR75-PELP1-MT cells and tumor growth was measured at weekly intervals. Left, tumor volume. B, average tumor weight. Representative images of tumors are shown. Status of PELP1 phosphorylation (C) and PCNA expression as a marker of proliferation (D) was analyzed by immunohistochemistry. *, $P < 0.05$; **, $P < 0.001$.

proliferation in the PELP1-WT xenograft tumors than in the PELP1-MT tumors (Fig. 5D). These results suggested that CDK phosphorylation of PELP1 is vital in tumorigenesis and growth of E2-driven tumor growth *in vivo*.

Discussion

E2 is known to promote key cell cycle events such as activation of CDK and hyperphosphorylation of pRb in ER-positive breast epithelial cells, leading to increased rate of G₁-S phase transition (8). Both CDK4 and CDK2 drive G₁-S transition in the cell cycle, and their expression is deregulated in tumors, indicating that phosphorylation of downstream effector proteins by CDKs is vital in tumorigenesis (3). We found that (a) PELP1 phosphorylation changes during cell cycle progression, (b) PELP1 interacts with the G₁-S phase CDKs (both CDK4 and CDK2), (c) PELP1 couples E2

signaling to the E2F axis, (d) PELP1 is a novel substrate of CDKs, and (e) CDK phosphorylation plays a key role in PELP1 oncogenic functions. Collectively, these results suggest that phosphorylation of PELP1 by CDKs confers a growth advantage to breast epithelial cells and thus contributes to tumorigenesis by accelerating cell cycle progression.

Our results identified ER coregulator PELP1 as another novel substrate of CDKs and that its phosphorylation is essential for optimal cell cycle progression. CDKs phosphorylate PELP1 minimally at two distinct sites (Ser⁴⁷⁷ and Ser⁹⁹¹), and these sites are phosphorylated by distinct CDK/cyclin complexes. We only validated Ser⁴⁷⁷ and Ser⁹⁹¹ as *in vivo* sites for CDK4 and CDK2, respectively. It is possible that there may be additional putative minor sites that could be phosphorylated *in vivo*, and we will explore those possibilities in future studies. Ser⁴⁷⁷ was previously identified as a site of phosphorylation of PELP1 in a large screen for epidermal growth factor

(EGF)-stimulated phosphoproteins (29), and interestingly, EGF is shown to activate CDK4/cyclin D1 complexes (30). We speculate that Ser⁴⁷⁷ could be the site that facilitates ER/CDK4 cross talk, which occurs during mammary gland development and also in pathologic settings including breast cancer progression. Reinforcement of Ser⁴⁷⁷ phosphorylation by CDK4/cyclin D1 and CDK2/cyclin A2, two kinases in two different cell cycle phases, is quite intriguing and may be important for unknown reasons.

Evolving evidence suggests that PELP1 may function as a large scaffolding protein, modulating gene transcription with protein-protein interactions. The NH₂ terminus of PELP1 interacts with ER α , pRb, and Src (12). Our results suggest that the NH₂ terminus of PELP1 also harbors a binding site for CDK4 that facilitates PELP1 phosphorylation. We found that during cell cycle progression, PELP1 runs as two to three slower-migrating bands and has a molecular weight of >160 kDa. Posttranslational modification of PELP1 probably accounts for the multiple shifts of PELP1 observed on denaturing gradient gels during cell cycle progression, as previously described for pRb (31). We also showed that PELP1-WT, but not PELP1-MT, overexpression promoted progression of breast cancer cells to S phase and that PELP1-MT significantly reduced E2-mediated *in vivo* tumorigenic potential. Our findings suggest that CDK phosphorylation of PELP1 plays a permissive role in E2-mediated cell cycle progression, presumably via its regulatory interaction with the E2F pathway.

Coregulators are often recruited by transcription factors to mediate epigenetic modifications at target gene promoters; for example, E2F uses coregulators such as HCF1 (32) and KAP1 (33) to facilitate gene activation and repression, respectively. PELP1 can interact not only with histone-modifying acetylases and deacetylases (34) but also with histone-modifying methyltransferases and demethylases. A recent study identified PELP1 as a component of the MLL1 methyltransferase complex (35), and we have found that PELP1 functions as a reader of dimethyl-modified histones (36). Our results from the cell proliferation assays using PELP1-MT cells established the significance and role of CDK phosphorylation of PELP1 in cell cycle progression. Collectively, these results suggest that PELP1 serves as a key coregulator that connects E2 signaling to the activation of E2F target genes

probably by facilitating epigenetic changes, which will be addressed in future studies.

PELP1 expression is deregulated in metastatic tumors (13). PELP1 protein expression is an independent prognostic predictor of shorter breast cancer-specific survival, and its elevated expression is positively associated with markers of poor outcome (14). Our data suggest that ER-CDK-PELP1 signaling plays a role in E2-mediated cell cycle progression and that the CDK deregulation commonly seen in breast tumors may play a role in metastasis by enhancing E2-mediated cell cycle progression via excessive phosphorylation of PELP1. Deregulation of both CDKs and PELP1 in breast cancer suggests that the modulation of PELP1 pathway by CDKs may represent a potential mechanism by which CDKs promote breast tumorigenesis.

In summary, our data provide the first evidence showing PELP1 as a novel substrate of CDKs. We also provide evidence to indicate that PELP1 phosphorylation by CDKs is essential for optimal E2-mediated cell cycle progression. On the basis of these findings, we predict that phosphorylation of PELP1 by CDKs confers a growth advantage to breast epithelial cells by activating the pRb/E2F pathway and thus contributes toward tumorigenesis by accelerating cell cycle progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Integrin-linked kinase 1: role in hormonal cancer progression

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1. ABSTRACT

Integrin-linked kinase 1 (ILK1) is a serine/threonine kinase that plays important roles in a variety of cellular functions including cell survival, migration and angiogenesis. ILK1 is normally expressed in numerous tissues and activated by growth factors, cytokines and hormones. Dysregulation of ILK1 expression or function is found in several hormonal tumors including breast, ovary and prostate. Emerging evidence suggests that ILK overexpression promotes cellular transformation, cell survival, epithelial mesenchymal transition (EMT), and metastasis of hormonal cancer cells while inhibition of ILK1 reduces tumor growth and progression. The recent development of ILK1 inhibitors has provided novel mechanisms for blocking ILK1 signaling to curb metastasis and therapy resistance of hormonal tumors. This review will focus on recent advances made towards understanding the role of ILK signaling axis in progression of hormonal cancer.

2. INTRODUCTION

ILK1 is a 59 kDa cytoplasmic protein that contains three distinct domains: (a) a phosphoinositide phospholipid-binding domain that mediates phosphoinositide binding, (b) an N-terminal ankyrin repeat domain that facilitates protein interactions and (c) a C-terminal serine/threonine protein kinase domain (1). ILK1 is a serine/threonine kinase that was first discovered as an integrin-binding protein in a yeast two-hybrid screen (2). It is able to directly activate several signaling pathways downstream of integrins and to participate in integrin signaling crosstalk with growth factors and hormones (3, 4). Substrates of the ILK1 include integrin β 1 (2), myosin light chain (MLC) (5), protein kinase B /Akt (AKT) and Glycogen synthase kinase 3 (GSK-3) (6). ILK1 is a unique kinase because it also functions as an intracellular adaptor protein, coupling a wide variety of signaling proteins to integrin and growth factor signaling. ILK interacting proteins include Pinch (7), Paxillin (8), Parvins (9), Affixin

ILK in hormonal cancer

(10), ILK BP (11), p21 activated kinase 1 (Pak1) (12) and estrogen receptor (ER) (13). Physiological signals including growth factors (6), cytokines (14, 15) estrogen (16), and the Wnt pathway (17) can activate ILK. Direct regulators of ILK include phosphoinositide 3-kinase (PI3K) (6), phosphatase and tensin homolog (PTEN) (18), protein phosphatase 2C (19), ILKAP (20) and secreted protein acidic and rich in cysteine (SPARC) (21). Accumulating evidence implicates ILK1 as a potential oncogene modulating several signaling pathways for cancer cell survival and tumor progression (22). Here, we will summarize key evidence for ILK signaling in hormonal tumor progression and discuss the possibility of the ILK1 axis as a possible therapeutic target for hormonal cancers.

3. DISCUSSION

3.1. ILK1 signaling and tumorigenesis

ILK1 signaling axis is implicated in many key signaling pathways that are activated in tumor cells promoting anchorage independence, motility, apoptosis, angiogenesis, EMT and tumor progression (22). Overexpression of ILK in epithelial cells enables anchorage-independent growth and survival of tumor cells (22-24) and tumorigenicity in nude mice (25). ILK1 overexpression in prostate cancer cells can suppress anoikis, promote anchorage-independence, and induce tumorigenesis (22). Accordingly, inhibition of ILK1 in prostatic adenocarcinoma (CaP) cells elicits cell cycle arrest and induces apoptosis (26).

Compared to normal cells, breast cancer cells appear to be preferentially dependent on the ILK1 signaling for survival (27). ILK1 function is also required for cytokine osteopontin (OPN)-induced AKT activation and for prostate cancer cell survival (28). Rictor, a known regulator of cytoskeletal dynamics, interacts with ILK1 to promote AKT phosphorylation leading to cell survival in cancer cells (29, 30). Transgenic mice expressing ILK1 in the mammary epithelium (MMTV-ILK1) develop a hyperplastic mammary phenotype and focal mammary tumors (31). These results provide strong evidence for an *in vivo* oncogenic role for ILK1 (31). Given the focal nature and long latency of the tumors, additional genetic events are likely required for tumor induction in MMTV-ILK1 mice.

ILK1-mediated AKT Ser473 phosphorylation may be celltype and context dependent. For example, genetic studies in *Drosophila Melanogaster* and *Caenorhabditis elegans* show AKT phosphorylation on Ser473 was not affected in ILK1 mutants (32, 33) and levels of Ser473 phosphorylation on AKT were equivalent in ILK1-null and wild-type mouse fibroblasts (34). The majority of analyses using tumor cells indicate that AKT Ser473 phosphorylation is dependent on a functional ILK1 axis; hence, the ILK1 pathway may be important during epithelial tumor progression presumably by promoting cell survival. In breast cancer cells, inhibition of ILK1 activity results in a decrease in AKT Ser473 phosphorylation and induction of apoptosis; whereas inhibition of ILK1 in normal cells has no such effects. These findings suggest

that ILK1 promotes survival function uniquely in breast cancer cells. ILK1 targeted treatment using specific ILK1 inhibitors may therefore have potential to reduce side effects in cancer patients (27).

Evidence also implicates ILK1 in regulating tumor angiogenesis; ILK1 increases vascular endothelial growth factor (VEGF), modulate levels of hypoxia inducible factor (HIF α)—blood-vessel formation and tumor growth of VEGF-treated endothelial cells (35, 36). In ovarian cancer cells, ILK1 serves as a key mediator in transforming growth factor (TGF) β 1 regulation of uPA/PAI-1 system, which is critical for the invasiveness of human ovarian cancer cells (37). ILK1 promotes epithelial to mesenchymal transformation (EMT) of cancer cells by modulating β -catenin/TCF, Snail and TGF β pathways (38-40). Collectively, these evolving findings indicate ILK1 signaling has the potential to activate multiple signaling pathways that contribute to the growth advantage of cancer cells.

3.2. Expression of ILK in hormonal cancers

While ILK1 is normally expressed in many hormonal tissues, emerging evidence implicates dysregulation of ILK1 expression and/or activity in many cancers including those of the breast, prostate and ovary (22). ILK1 expression increases as ovarian tumor grade and its expression can be sustained by peritoneal tumor fluid (PTF). PTF-induced over-expression of ILK correlates with the activation of the AKT pathway (41). Thus, ILK1 has potential to serve as a biological marker for early detection and a therapeutic target for ovarian cancer (41).

One study found that serum from ovarian cancer patients contains cell-free immunoreactive ILK1 at statistically elevated levels compared to controls without ovarian cancer (42); ILK1 was present at elevated levels in both the serum and PTF of ovarian cancer patients. The correlation between ILK1 expression with CA125 concentration in these biological fluids suggests a potential role of ILK1 as a serological ovarian tumor marker for early detection and treatment monitoring (43). Integrin α 5 β 1 upregulates ILK1 expression in human ovarian cancer cells via enhancement of ILK1 gene transcription. Mechanistic studies show that transcription factor Ets contributes to α 5 β 1-mediated ILK1 upregulation. By increasing ILK1 as an important integrin-proximal kinase, α 5 β 1 may promote its intracellular signaling and tumor biological processes (42).

ILK1 mRNA is upregulated in prostate adenocarcinoma cells compared to normal epithelial cells and therefore, can be a useful internal reference gene marker (44). ILK1 expression also increases with prostate tumor grade and is specifically associated with the increased proliferative index that typifies CaP progression. Further, enhanced ILK1 expression is inversely related to 5-year patient survival linking increased ILK1 expression in prostate tumor progression (26). b-parvin (ParvB) is an adaptor protein that binds to the ILK1. Expression studies indicated ParvB expression was down regulated in breast tumors compared to ParvB expression in patient-matched

normal mammary gland tissue. These results suggest that loss of ParvB expression could be a mechanism for upregulating ILK1 activity in tumors (9).

3.3. Role of ILK1 in cancer cell metastasis

Metastasis is a frequent and fatal culmination to hormone-sensitive cancers, particularly of ovarian origin. Acquisition of invasive and migratory characteristics in cancer cells results primarily from adopting an EMT phenotype. This phenotype is supported by various pro-metastatic factors. Emerging studies have unraveled the role of ILK1 in governing metastatic features in various cancers, predominantly for acquiring the mesenchymal phenotype and promoting cell invasion through increased expression of various matrix-degrading proteases.

Increased expression of ILK1 correlates significantly with higher grade of ovarian tumors (41, 43). ILK1 played a predominant role in endothelin-1 (ET-1/ETAR)-induced EMT and in the development of the invasive phenotype in ovarian cancer by increasing levels of Snail, stabilizing beta-catenin and suppressing E-cadherin expression through a PI3K-dependent signaling pathway. Also, enhanced expression and activity of matrix metalloproteinases (MMP-2 and MMP-9) mediated by ET-1 correlated with increased ILK expression (45). In addition, PI3K-ILK1 axis played a critical role in TGF β 1-mediated invasive phenotype in ovarian epithelial cancer cells via up-regulation of urokinase-type plasminogen activator (uPA) and PA inhibitor 1 (PAI-1). It is worthwhile to note that expression of uPA and PAI-1 were reported to correlate with advanced stages of ovarian cancer (46). Unlike ET-1, the TGF β 1-mediated increase in MMP-2 expression was found to be independent of ILK1 signaling (37). Similarly, Y-box-binding protein 1 (YB-1), a known, poor prognostic marker of ovarian cancer, localized in the nucleus and enhanced CXCR4 expression for acquiring the malignant phenotype (47). Interestingly, siRNA depletion of ILK1 and AKT affects both nuclear translocation of YB-1 and expression of CXCR4 in ovarian cancer cells, suggesting that disrupting ILK1-AKT pathway can be used to block YB-1-mediated metastasis (48).

One of the earliest insights into the potential of ILK1 to govern the metastatic phenotype came from the Dedhar lab. They demonstrated that stable overexpression of ILK1 in scp2 murine mammary gland epithelial cell lines induced the classic EMT phenotype including reduction in E-cadherin along with translocation of β -catenin and formation of β -catenin/LEF complex inside the nucleus and thus upregulating expression of various mesenchymal genes (49). Similarly, Somasiri *et al.*, found that forced exogenous expression of wild type ILK1 but not the dominant negative kinase-dead version of ILK in scp2 murine mammary epithelial cell lines induced the EMT phenotype via reduction in E-Cadherins and acquisition of vimentin filaments (39). Suppression of anoikis, a unique process of apoptosis resulting from insufficient cell-matrix adhesion, appears to be an important event in the development of metastasis (50). Studies using both scp2 murine mammary cell lines and human breast cancer cell lines implicated ILK1 as a suppressor of anoikis (24).

Inhibition of cell death/anoikis by ILK overexpression supports the idea that ILK is a predominant player in regulating the emergence of the metastatic phenotype. Subsequent to these studies, ILK-mediated induction of the invasive phenotype in mammary epithelial cells was found to be associated with increased MMP-9 expression. This increase in MMP-9 proteins was attributed to ILK-mediated activation of GSK-3 β and AP-1 transcription factor (51). The ILK1-AP1 axis was further shown to contribute to the invasive phenotype in mammary gland epithelial cells mediated by osteopontin (OPN), a metastasis-associated glyco-phosphoprotein. Using the murine metastatic mammary epithelial cells 4T1, Mi *et al.*, demonstrated that an OPN-mediated increase in expression of MMP2 and uPA can be attributed to ILK1-dependent AP-1 activation (14). Transgenic mice specifically expressing ILK in mammary glands had increased mesenchymal-like cell populations within their tumors, suggesting that stand-alone ILK1 overexpression can initiate the EMT phenotype (31). Another piece of evidence connecting ILK1 to anoikis is from a study that demonstrated the role of the tumor suppressor DOC-2/hDab-2 in the induction of anoikis in breast cancer cells. DOC-2 was shown to induce anoikis by down regulating ILK activity but this activity was found to be independent of the PI3K/AKT and MAPK pathways, suggesting that ILK1 may utilize alternate pathways to suppress anoikis and promote anchorage independence (52). Estrogen-mediated extranuclear functions are also shown to activate ILK1. Since PELP1 expression is upregulated in metastatic breast cancer (53); modulation of the ILK1 pathway by PELP1 may represent a potential mechanism by which estrogen signaling promotes metastasis in breast cancer cells (54).

Relatively fewer studies have been done to elucidate the role of ILK1 in metastatic prostate, cervical and endometrial cancers. A recent study using prostate cancer cell lines implicated ILK1 as a downstream effector for talin1-mediated resistance to anoikis (55). It is worthy to note that talin1 was found to be overexpressed in metastatic prostate cancer and tumors with a high Gleason score when compared to its expression in normal Gleason scores and benign tumors (55). PI3K/AKT-dependent anoikis has also been found in endometrial cancer cell lines but the role of ILK1 in governing this anoikis has not yet been demonstrated (56). Similarly, Notch1-Rhoc axis and anoikis are reported to regulate the metastatic potential in cervical cancer progression but the role of ILK1 in these cancers remains elusive (57).

3.4. ILK and ER signaling crosstalk

Several lines of evidence implicate ILK1 axis crosstalk with ER signaling. One of the lacunae in our understanding of the mechanistic details of the metastatic evolution of these hormonal cancers and how hormones like estrogen and their respective steroid receptors regulate ILK1 pathway. Dr. Kumar's group provided first evidence of estrogen receptor (ER)-ILK crosstalk. They showed a direct interaction of ER with ILK1 and that the interaction occurred through the nuclear receptor box (i.e., LXXLL) located between the ILK1 pleckstrin homology-like domain and the ankyrin repeats (58). In addition, we

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recently identified ILK1 as a novel interacting protein of PELP1, an ER-coregulator protein (54). Our study demonstrated that ILK functions as a downstream effector of ER extranuclear signaling, leading to cytoskeleton reorganization. These extranuclear actions of estrogen facilitated activation of the ILK enzyme via the PI3K pathway and inhibition of ILK functions significantly affected the estrogen-mediated cell migratory potential. The proposed signaling pathway is E2>PELP1>PI3K>ILK>CDC42 and it may contribute to estrogen mediated cytoskeleton changes (54). Earlier evidence suggests that the ILK1 axis is a major signaling node that links integrins and growth factor signaling to a variety of cellular responses. The ability of ILK1 to interact with ER and growth factor/integrin signaling components suggests that deregulation of ILK has the potential to promote ER growth factor crosstalk and thus a potential to contribute to therapy resistance.

3.5. ILK1 and hormonal therapy resistance

Deregulation of human epidermal growth factor receptor 2 (ErbB2) expression and /or signaling has emerged as the most significant factor in the development of hormonal resistance (59). ErbB2 is an oncogene that has been shown to be over expressed, amplified, or both, in several human malignancies including breast tumors. ER expression occurs in ~50% ErbB2 positive breast cancers and crosstalk between the ER and ErbB2 pathways promotes endocrine therapy resistance (58, 59). Disruption of ILK expression by siRNA or inhibition of ILK1 function in ErbB2-expressing cells with a small molecule inhibitor resulted in a profound block in invasive properties resulting from the induction of apoptotic cell death. These observations support the concept of ILK1 having a critical role in the initiation phase of ErbB2 tumor induction (61).

AKT signaling plays an important role in the development of hormonal therapy resistance (62). Many hormonal tumors exhibit an increase in constitutively active AKT; however, mutations in AKT are rare in breast tumors (62). Therefore, proteins contributing to AKT activation may play a role in the development of therapy resistance. In this context, several lines of evidence indicate that ILK is a receptor-proximal effector for the PI3K-dependent, extracellular matrix- and growth factor-mediated activation of PKB/AKT and inhibition of GSK-3 (6). Since ILK1 expression is deregulated in hormonal cancer, increases in ILK1 signaling has the potential to contribute to therapy resistance.

Nuclear localization of PAK1, a proto-oncogene (63), is associated with the progressive limitation of tamoxifen sensitivity and implicated in development hormonal therapy resistance (64). ILK1 is a PAK1 substrate, and undergoes phosphorylation-dependent shuttling between the cell nucleus and cytoplasm, and interacts with gene-regulatory chromatin, thus ILK1-PAK1 interactions may have a role in therapy resistance (12).

Cyclin D1 overexpression commonly occurs in breast cancer. The level of cyclin D1 expression and activated STAT3 are important markers to predict response

to tamoxifen treatment (65). ILK1 signaling increases cyclin D1 protein levels (65). Mechanistic studies showed that ILK-induced CREB transactivation and CREB binding to the cyclin D1 promoter CRE led to cyclin D activation. Wnt-1, an oncogene implicated in mammary tumorigenesis also induced cyclin D1 mRNA via ILK pathway (65).

ER-coregulators play an essential role in hormonal therapy responsiveness and cancer progression (67). Recent findings suggest that ILK1 interacts with ER-coregulator PELP1 (54) and that such interactions enhance ILK1-kinase activity. Since PELP1 expression is commonly deregulated in many hormone-responsive tissues (16), the PELP1-ILK1 interaction is likely to have significant implications in tumor cell survival and therapy resistance.

3.6. Therapeutic potential of targeting ILK in hormonal cancers

Current strategies to block the ILK1-mediated phenotype using *in vitro* systems include the usage of small molecular inhibitors (like KP-392/KP-SD-1 and QLT-0267) (27, 57, 68) and ILK-targeted siRNAs and antisense oligonucleotides (69). Also, the use of a dominant negative ILK (ILK-E359K) has been proposed. Initial work towards generating potent small molecule inhibitors against ILK1 was accomplished by Dedhar and colleagues in collaboration with Kinetek Pharmaceuticals (now a part of QLT Inc). As a result the KP-392/KP-SD-1 and KP-SD-2 compounds were identified and shown to inhibit ILK-mediated AKT activation and the EMT phenotype when tested using *in vitro* model cells. The use of KP-SD-1 was highly encouraging when tested on human colon carcinoma cells using an *in vivo* xenograft transplantation assay (70). QLT-0267 is a second-generation ILK1 inhibitor that is more potent with increased sensitivity over the parental KP-392 compound (27). Interestingly, QLT-2067 induces apoptosis in the breast cancer cell lines MDA-MB-231, MDA-MB-435, BT-549, and MDA-MB-468, but not in normal human breast epithelial cells at a concentration of 10 μ mol/L or less.

Some evidence indicates blockage of ILK1 signaling along with conventional chemotherapy may be beneficial. QLT-0267 in combination with docetaxel exhibited synergistic effects on reducing the viability of various breast cancer cells (68). A remarkable observation made in this study was that the ErbB2 status of cells has a definitive effect on this combinatorial treatment. Low ErbB2-expressing cells were more sensitive to this combination when reduction of phospho-AKT was used as endpoint for assessing the efficacy. Further, this combination was found to be more effective than the single treatment in reducing the tumor burden and prolonging survival in an orthotopic breast cancer model using transplanted LCC6 cells, which have reduced ErbB2 expression. Similarly, the ILK1 inhibitor KP-307-2, an analog of KP-392, was found to suppress tumorigenesis in xenograft tumor models using the prostate cancer cell line PC3. Surprisingly, a novel feedback mechanism between ILK and VEGF expression was also observed in this study and therefore treatment with ILK1 inhibitor causes a

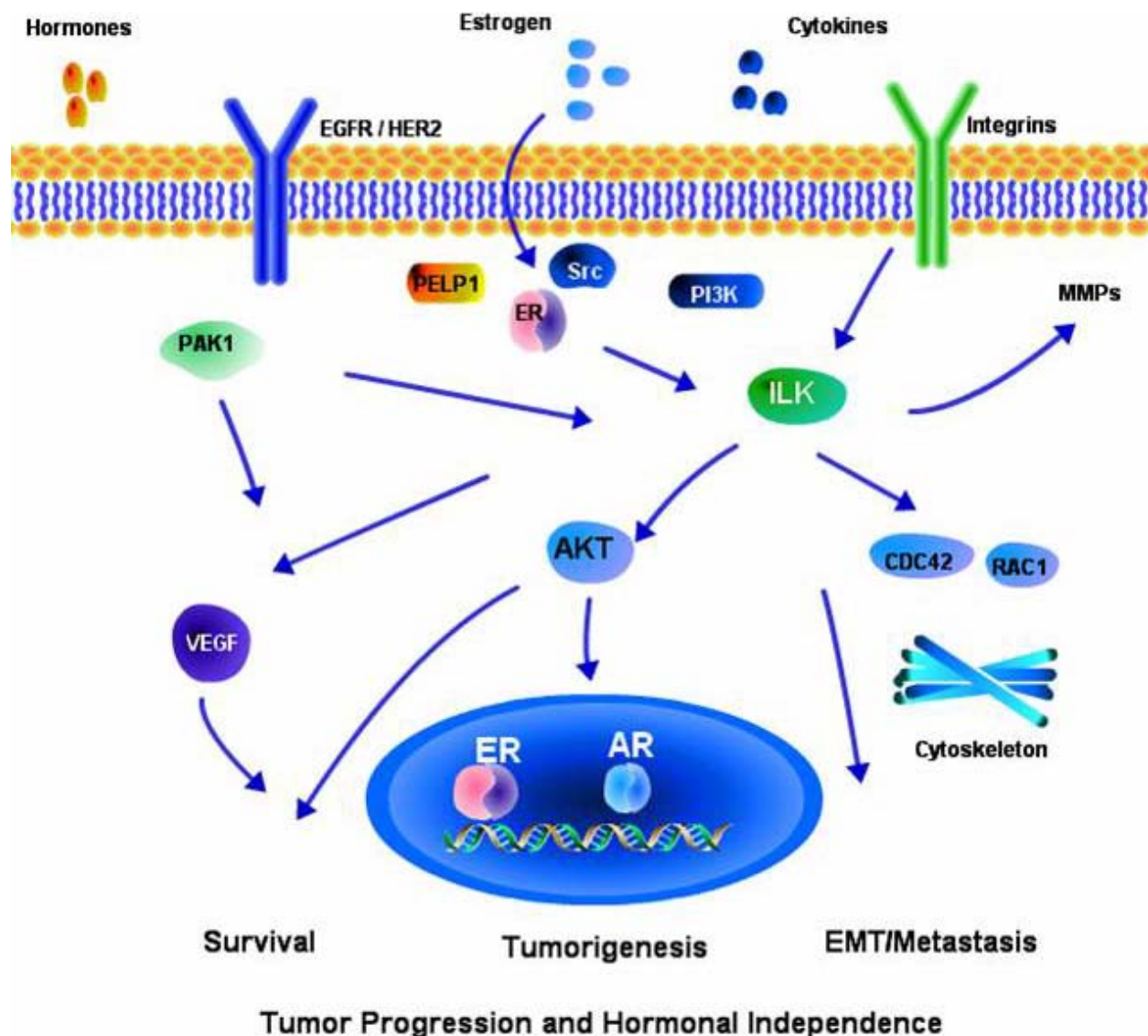


Figure 1. Schematic representation of the current understanding of ILK1 signaling crosstalk with pathways that are commonly deregulated in hormonal cancers. Crosstalk of ILK axis with the estrogen receptor, ERBB2, Pak1 signaling pathways suggest that deregulation of ILK expression and/or function is likely to contribute to the hormonal cancer progression and development of therapy resistance.

‘double jeopardy’ situation in the cells by causing inhibition of tumorigenesis and suppressing angiogenesis (35).

The second generation ILK1 inhibitor QLT-0267 may be useful in radiosensitizing cancer cells, particularly squamous cell carcinoma cells of head and neck and also, engenders the possibility of similar effect on gynecologic cervical carcinoma that is predominantly squamous cell carcinoma upon histological type (71). Overall, it appears that ILK1 inhibitors, although at various levels of development, have the potential to down regulate ILK1 activity, the ILK1-mediated EMT phenotype and

tumorigenesis when tested using *in vitro* and various pre-clinical mouse models.

4. FUTURE PERSPECTIVES

In summary, the data reviewed herein provides support for the following conclusions:(a) deregulation of ILK1 expression and/or functions occurs in human hormonal cancers; (b) inhibition of ILK1 correlates with delayed tumor growth in preclinical models; (c) ILK1 can modulate key signaling pathways including cell survival, tumor growth, angiogenesis, EMT and metastasis; and (d) ILK1 crosstalk with various signaling pathways that are commonly deregulated in hormonal cancers including

ErbB2, PAK1 and ER (Figure 1). Thus, these data strongly support a role of ILK1 in the hormonal cancer progression. The recent availability of drugs that specifically target ILK1 has begun to open up new avenues for targeting hormonal tumors. Most interesting is the difference in sensitivity to the effects of ILK inhibition between normal breast epithelial and breast cancer cells, provide a potential for the use of ILK1 inhibitors in patient therapy. Future studies using combination of ILK inhibitors with drugs that target hormone therapy could possibly be done to achieve significant reduction in various hormonal cancers. Future studies are warranted to identify the signaling pathways that regulate ILK1 expression in hormonal cancers and to examine the prognostic / diagnostic significance of ILK1 using larger number of tumor samples. A better understanding of the ILK1 signaling and its crosstalk with hormonal signaling components is expected to assist in the development of an integrated model for targeting ILKs in the management of hormone-driven tumors.

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Abbreviations: AKT: protein kinase B; EMT:epithelial mesenchymal transition; ErbB2: epidermal growth factor receptor2; ER: estrogen receptor; ILK1: integrin linked kinase 1; Pak1:p21 activated kinase 1; ; PI3K: phosphatidylinositol 3 kinase. TGF:transforming growth factor.

Key Words: Estrogen receptor, hormonal signaling, therapy resistance, integrin linked kinase, EMT, metastasis, AKT, ErbB2.

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ABSTRACT

1. **Nair BC**, Vallabhaneni S, Li R, Tekmal RR, Vadlamudi RK (2010). Roscovitine confers tumor suppressive effect on therapy-resistant breast tumor cells. *Presented at 33rd Annual San Antonio Breast Cancer Symposium.*

[P5-06-12] Roscovitine Confers Tumor Suppressive Effect on Therapy-Resistant Breast Tumor Cells.

Nair BC, Vallabhaneni S, Li R, Tekmal RR, Vadlamudi RK. The University of Texas Health Sciences Center at San Antonio

Background: Current clinical strategies for treating hormonal breast cancer involve the use of antiestrogens (AE) that block ER α functions, and aromatase inhibitors (AI) that decrease local and systemic estrogen production. *Both of these strategies improve outcomes for ER+ve breast cancer; however development of therapy resistance is a major clinical problem.* Emerging data implicate ER-signaling cross talk, and alteration in ER subtypes as the major causes of resistance. Current evidence suggest that ER α increases proliferation, while ER β decreases cell proliferation and the ratio between ER α and ER β is the driving force for tumor cell proliferation. Further, most downstream events in the resistance pathways converge upon modulation of cell cycle regulatory proteins; the most conspicuous of which is the activation of Cyclin Dependent Kinase 2 (CDK2) pathway. Roscovitine is one of the most frequently studied and used CDK2 inhibitor. In this study, we examined whether Roscovitine confers tumor suppressive advantage to therapy resistant breast epithelial cells by inhibiting CDK functions.

Methods: We tested the effect roscovitine using three different therapy resistant model cells; (a) MCF-7-Tam (acquired Tamoxifen resistance model); (b) MCF-7-LTLT-ca (acquired Letrozole resistance model); (c) MCF-7-HER2 that exhibit Tamoxifen resistance (ER-growth factor signaling cross talk model). Cells were treated with Roscovitine (0-30 μ M) for 24 hours and proliferation was measured after 48h using a luminescent cell viability assay (Promega). CDK activity, expression of ER α , β subtypes, ER-coregulators, and expression of ER target genes was measured by Western and PCR assays. *To test the efficacy of Roscovitine in vivo*, pre-clinical nude mice models bearing xenografts of therapy resistant cells were subjected to orally available Roscovitine (100mg/kg/tid/oral) for 10 days or treated with vehicle and tumor growth was monitored for a further 15 days.

Results: The results showed that Roscovitine (10 μ M) significantly inhibited the growth of all three therapy resistant cells in cell proliferation and foci formation assays ($P < 0.05$). FACS analysis revealed that Roscovitine treatment increased proportion of cells in G2-M phase. Therapy resistant cells treated with Roscovitine showed decreased CDK activity and low cyclin D levels indicating decreased cell proliferation. Interestingly, these studies also revealed an unexpected discovery that CDK inhibitor Roscovitine has potential to alter the ratio of Estrogen receptors with preferential upregulation of ER β with concomitant down regulation of ER α , ER-coregulators including AIB1 and PELP1. Results from xenograft studies showed that Roscovitine substantially reduced growth of therapy resistant tumors.

Conclusions: Our results suggest that Roscovitine can be used to inhibit the growth of therapy resistant cells. Since many advanced and therapy resistant tumors exhibit loss or reduced expression of ER β , dual action of Roscovitine will provide a novel drug to inhibit both CDKs and to increase the expression of tumor suppressor ER β , thus has potential to serve as a double-edged sword to interfere

with the resistance mechanisms. Supported by DOD Fellowship W81XWH-09-1-0010 (BCN) and NIH grant CA095681(RV).

Saturday, December 11, 2010 5:30 PM

**Poster Session 5: Tumor Cell and Molecular Biology: Novel/Emerging Therapeutic Targets
(5:30 PM-7:30 PM)**

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ABSTRACT

2. **Nair BC**, Morales M, Nair HB, Vallabhaneni S, Li R, Tekmal RR, Vadlamudi RK (2010). Roscovitine modulates ER α /ER β ratio: A novel means to target hormonal therapy resistance. *Proceedings of the 101st Annual Meeting of the American Association for Cancer Research; 2010 Apr 17-21; Washington, DC. Philadelphia (PA): AACR; 2010*

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Presentation Abstract

Abstract
Number: 4604

Presentation
Title: **Roscovitin modulates ER α /ER β ratio: A novel means to target hormonal therapy resistance**

Presentation
Time: Tuesday, Apr 20, 2010, 2:00 PM - 5:00 PM

Location: Exhibit Hall A-C, Poster Section 29

Poster
Section: 29

Poster
Board
Number: 15

Author
Block: Binoj Chandrasekharan Nair, Mary Morales, Hareesh Babu Bhaskaran Nair, Sreeram Vallabhaneni, Rong Li, Rajeshwar Rao Tekmal, Ratna K. Vadlamudi. UT Health Science Ctr., San Antonio, TX

Abstract
Body: Current endocrine therapy for ER+ve breast cancer involves modulating ER α -pathway using either ER α -antagonists or aromatase inhibitors. Despite the positive effects, de novo and/or acquired resistance to endocrine therapies frequently occur. Although mechanisms for hormonal therapy resistance remains elusive, emerging data implicate ER-growth factor signaling cross talk, and alteration in ER subtypes as the major causes of resistance. Most downstream events in the resistance pathways converge upon modulation of cell cycle regulatory proteins; the most conspicuous of which is the activation of Cyclin Dependent Kinase 2 (CDK2) pathway. Roscovitin is one of the most frequently studied and used CDK2 inhibitor. In this study, we examined whether roscovitin confers tumor suppressive advantage to therapy resistant breast epithelial cells by inhibiting CDK functions using three therapy resistant model cells; (a) MCF-7-Tam (acquired Tamoxifen resistance model); (b) MCF-7-CA-LTLT (acquired Letrozole resistance model); (c) MCF-7-HER2 (ER-growth factor signaling cross talk model). Roscovitin at 10 μ M concentration substantially reduced the growth of all three resistant model cells in cell proliferation and foci formation assays. FACS analysis revealed that roscovitin treatment increased proportion of cells in G2-M. To increase the drug efficacy, we have developed PLGA nanoparticles containing roscovitin and this mode of delivery significantly reduced the dosage needed to see the growth inhibitory effect in therapy resistant cells to 500 nM. To determine the mechanism, we have determined the relationship between CDK activity, expression of ER α , β subtypes, ER-coregulators, and expression of ER target genes in the presence or absence of roscovitin in resistant cells. Roscovitin substantially affected CDK2 activity and decreased cyclin A levels in the model cells. These studies also revealed an unexpected discovery that CDK inhibitor roscovitin has potential to alter the ratio of ER isoforms with preferential upregulation of ER β expression with concomitant down regulation of ER α , its coregulators including AIB1 and PELP1. Since many advanced and therapy resistant tumors exhibit loss or reduced expression of ER β , dual action of roscovitin will provide a novel drug to inhibit both CDKs and to increase the expression of tumor suppressor ER β . Since deregulation of cell cycle machinery and estrogen receptor signaling contributes to resistance, roscovitin a drug that attacks both these pathways will serve as a double-edged sword to interfere with the resistance mechanisms.

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